UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Papel de las N-aciletanolaminas y el sistema circadiano en la homeostasis energética en peces teleósteos

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Miguel Gómez Boronat

Directoras

Nuria de Pedro Ormeño
Esther Isorna Alonso

Madrid

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Papel de las N-aciletanolaminas y el sistema circadiano en la homeostasis energética en peces teleósteos

Miguel Gómez Boronat

Directoras: Nuria de Pedro Ormeño y Esther Isorna Alonso

Madrid, 2019

UNIVERSIDAD COMPLUTENSE MADRID

DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD DE LA TESIS PRESENTADA PARA LA OBTENCIÓN DEL TÍTULO DE DOCTOR

D./Dña. MIGUEL GÓMEZ BORONAT

con número de DNI/NIE/Pasaporte 72890535-Q, estudiante en el Programa de Doctorado EN BIOLOGÍA,

de la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid, como autor/a de la tesis presentada para la obtención del título de Doctor y titulada:

PAPEL DE LAS N-ACILETANOLAMINAS Y EL SISTEMA CIRCADIANO EN LA HOMEOSTASIS ENERGÉTICA EN PECES TELEÓSTEOS

y dirigida por: NURIA DE PEDRO ORMEÑO y ESTHER ISORNA ALONSO

DECLARO QUE:

La tesis es una obra original que no infringe los derechos de propiedad intelectual ni los derechos de propiedad industrial u otros, de acuerdo con el ordenamiento jurídico vigente, en particular, la Ley de Propiedad Intelectual (R.D. legislativo 1/1996, de 12 de abril, por el que se aprueba el texto refundido de la Ley de Propiedad Intelectual, modificado por la Ley 2/2019, de 1 de marzo, regularizando, aclarando y armonizando las disposiciones legales vigentes sobre la materia), en particular, las disposiciones referidas al derecho de cita.

Del mismo modo, asumo frente a la Universidad cualquier responsabilidad que pudiera derivarse de la autoría o falta de originalidad del contenido de la tesis presentada de conformidad con el ordenamiento jurídico vigente.

En Madrid, a 10 de julio de 20 19

Fdo.:

Esta DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD debe ser insertada en la primera página de la tesis presentada para la obtención del título de Doctor

COMPLUTENSE UNIVERSITY OF MADRID

FACULTY OF BIOLOGICAL SCIENCES

Department of Genetic, Physiology, and Microbiology
Teaching Unit of Animal Physiology

PhD THESIS

Role of N-acylethanolamines and the circadian system in the energy homeostasis in teleost fish

Thesis defended to obtain the

Doctorate Degree in Biology with International Mention by

Complutense University of Madrid

Miguel Gómez Boronat

Madrid, 2019

Certificado

Doña **NURIA DE PEDRO ORMEÑO**, Profesora Titular de la Universidad Complutense de Madrid, y Doña **ESTHER ISORNA ALONSO**, Profesora Titular de la Universidad Complutense de Madrid,

CERTIFICAN:

Que la presente Tesis Doctoral titulada "**Papel de las N-aciletanolaminas y el sistema circadiano en la homeostasis energética en peces teleósteos**" presentada por **Miguel Gómez Boronat**, licenciado en Biología, para optar al grado de **Doctor en Biología con Mención Internacional** por la **Universidad Complutense de Madrid**, ha sido realizada bajo su dirección y reúne los requisitos necesarios para proceder a su presentación y defensa pública.

Y para que así conste a los efectos oportunos, firmamos el presente certificado en Madrid, a 10 de julio de 2019.

Dra. Nuria de Pedro Ormeño Dra. Esther Isorna Alonso

Financiación

La presente Tesis Doctoral ha sido llevada a cabo en el Departamento de Genética, Fisiología y Microbiología (Unidad Docente de Fisiología Animal) de la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid, bajo la codirección de la Dra. Nuria de Pedro Ormeño y la Dra. Esther Isorna Alonso.

Este trabajo se realizó con ayuda del apoyo económico del Ministerio de Economía, Industria y Competitividad (proyectos AGL2013-46448-C3-2R y AGL2016-74857-C3-2R). Miguel Gómez Boronat ha sido beneficiario de una beca de Formación de Personal Investigador (FPI; BES-2014-068103) del Ministerio de Economía y Competitividad del Gobierno de España.

La presente Tesis Doctoral ha sido realizada y consecuentemente será defendida de forma adecuada para la obtención del titulo de **Doctor en Biología con Mención Internacional por la Universidad Complutense de Madrid**.

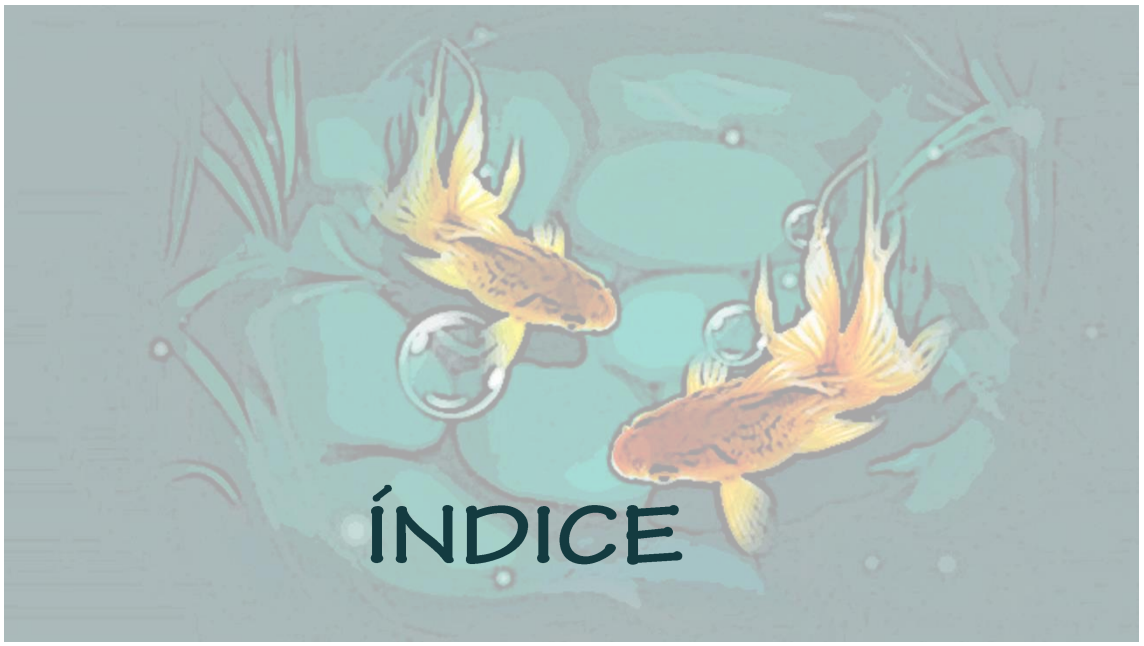
La presente Tesis Doctoral ha sido informada por dos expertos doctores pertenecientes a alguna institución de educación superior o instituto de investigación no española:

- **Ana Guijarro Antón**

  Dipartimento di Oncologia Cellulare
  Università di Genova & IRCCS AOU San Martino – IST Istituto Nazionale per la Ricerca sul Cancro
  Largo Rosanna Benzi 10
  16132 Genova, Italia

- **Catarina Cortes Valente de Oliveira**

  Centro de Ciências do Mar (CCMAR)
  Aquaculture Research Group
  Universidade do Algarve
  Gambelas Campus
  8005-139 Faro, Portugal



ÍNDICE

ÍNDICE 1

RESUMEN / SUMMARY 5

INTRODUCCIÓN 15

1. REGULACIÓN DE LA INGESTA EN PECES **17**

1.1. Regulación homeostática **18**

1.1.1. Señales hipotalámicas **19**

1.1.2. Señales gastrointestinales **21**

1.2. Regulación hedónica **23**

1.3. Regulación metabólica **24**

2. N–ACILETANOLAMINAS **26**

2.1. Estructura de los ligandos **26**

2.2. Síntesis, distribución y degradación **27**

2.3. Receptores **31**

2.4. Actividad biológica **33**

3. SISTEMA CIRCADIANO **36**

3.1. Base molecular de los relojes endógenos **39**

3.2. Organización funcional del sistema circadiano **43**

3.3. Interacciones con la homeostasis energética. Importancia de los receptores nucleares del bucle auxiliar **45**

4. EL CARPÍN: CARACTERÍSTICAS DE LA ESPECIE **48**

OBJETIVOS **51**

RESULTADOS **55**

CAPÍTULO 1. Papel de las N-aciletanolaminas en la homeostasis energética **57**

1.1. Efectos del factor de saciedad oleiletanolamida en el metabolismo hepático de los lípidos y de la glucosa en el carpín
***Journal of Comparative Physiology B* (2016) 186:1009–1021** **63**

1.2. Primeras evidencias acerca del papel de la palmitoiletanolamida sobre la homeostasis energética en peces
***Hormones & Behavior* (2019) En revisión** **77**

1.3. Perfiles diarios de las N-aciletanolaminas en el cerebro y tracto gastrointestinal del carpín: posible papel de la alimentación
***Frontiers in Neuroscience* (2019) 13:450** **87**

CAPÍTULO 2. Sistema circadiano y homeostasis temporal **111**

2.1. Ritmos diarios de receptores nucleares y genes reloj en el hipotálamo y el hígado del carpín bajo diferentes condiciones de luminosidad ***Chronobiology International*** **(2019) En revisión** **117**

2.2. Un desfase en el horario de la alimentación actúa como un agente estresante que altera los osciladores circadianos en el carpín ***Frontiers in Physiology*** **(2018) 9:1749** **137**

2.3. Influencia del horario de alimentación sobre los receptores nucleares PPARα y REV-ERBα en el hipotálamo y el hígado de carpín **151**

DISCUSIÓN **153**

1. PAPEL DE LAS N–ACILETANOLAMINAS EN LA HOMEOSTASIS ENERGÉTICA **155**

1.1. El sistema de las NAEs en el carpín **155**

1.2. Las NAEs como señales postprandiales de saciedad **157**

1.3. Regulación del metabolismo hepático por las NAEs **160**

2. RITMICIDAD DE SEÑALES RELACIONADAS CON LA HOMEOSTASIS ENERGÉTICA **164**

2.1. Sincronización de los osciladores por los *zeitgeber*s externos: ciclos luz-oscuridad y alimentación-ayuno **164**

2.2. Ritmos de NAEs y receptores nucleares **169**

CONCLUSIONES / CONCLUSIONS **175**

BIBLIOGRAFÍA **183**

RESUMEN / SUMMARY

RESUMEN

PAPEL DE LAS N-ACILETANOLAMINAS Y EL SISTEMA CIRCADIANO EN LA HOMEOSTASIS ENERGÉTICA EN PECES TELEÓSTEOS

INTRODUCCIÓN

La ingestión de alimentos es un proceso multifactorial en el que intervienen una gran cantidad de elementos tanto ambientales como fisiológicos, cuya regulación se encuentra muy conservada a lo largo de la evolución, desde peces teleósteos hasta mamíferos. Así, en todos los vertebrados, tanto el sistema nervioso central como una gran cantidad de órganos periféricos funcionan de forma coordinada para establecer una óptima regulación de la ingesta.

Actualmente se conocen un gran número de señales neuroendocrinas implicadas en la regulación de la homeostasis energética en los peces, como neuropéptidos, hormonas y monoaminas. Sin embargo, se ha prestado poca atención a moléculas de otra naturaleza, como los derivados lipídicos N-aciletanolaminas (NAEs), las cuáles además se han propuesto como candidatas a actuar como nexo entre el metabolismo y el sistema circadiano en los mamíferos, a través de su receptor PPARα.

Por todo ello, el objetivo general de la presente Tesis Doctoral es el estudio del papel de las NAEs en la homeostasis energética de los peces e investigar la relación entre el sistema circadiano y dicha homeostasis energética, utilizando como modelo el ciprínido *Carassius auratus*.

OBJETIVOS Y RESULTADOS

- **Caracterizar componentes esenciales del sistema de las NAEs en los peces**

Se ha demostrado la presencia en el carpín de todos los componentes del sistema de las NAEs: OEA, PEA y SEA, sus precursores, las enzimas de síntesis (NAPE-PLD) y de degradación (FAAH) y su receptor (PPARα) en diferentes tejidos centrales (hipotálamo y telencéfalo) y gastrointestinales (bulbo intestinal, intestino anterior e hígado).

- **Determinar el papel de las NAEs en la regulación de la ingesta en los peces**

La ingestión de alimento induce un aumento en los niveles gastrointestinales, pero no cerebrales, de OEA, PEA y SEA. Además, la PEA reduce la ingesta, el peso corporal y la actividad locomotora, junto con un aumento en la expresión génica hepática de *leptina* y una reducción en la expresión hipotalámica de *npy*.

- **Estudiar los posibles efectos de la OEA y la PEA sobre el metabolismo hepático e interacción con el sistema circadiano**

Tanto la OEA como la PEA producen un aumento de la actividad de enzimas lipogénicas (ACLY y FAS), junto con una reducción en la actividad de la enzima lipolítica CPT-1 por la OEA. Ambas NAEs también reducen la actividad de la enzima PEPCK y aumentan la de la GPasa. Asimismo, ambas NAEs modulan la expresión de algunos genes reloj en el hígado, destacando un aumento de la expresión de *bmal1a*. Los niveles de OEA, PEA y SEA, así como la expresión de *pparα*, en el hígado y el intestino presentan ritmos diarios asociados al momento de la ingesta.

- **Averiguar si el horario de alimentación y/o el ciclo de luz-oscuridad determinan la sincronización de los osciladores en peces**

Tanto en el hipotálamo como en el hígado los genes reloj presentan ritmos diarios de expresión. Dichos ritmos en el hipotálamo se pierden en ausencia del ciclo luz-oscuridad, mientras que no se ven afectados por cambios en el horario de alimentación. En el hígado, estos ritmos se mantienen incluso en oscuridad constante, ajustándose al horario de alimentación.

- **Comprobar si existen ritmos diarios de los receptores nucleares PPAR, ROR y REV-ERB, y averiguar qué *zeitgeber*s dirigen tales ritmos.**

La expresión rítmica de *rev-erbα* se encuentra controlada por el ciclo luz-oscuridad en el hipotálamo y por el horario de alimentación en el hígado. Por su parte, *rev-erbβ*, *rorα* y *pparα*

presentan ritmos diarios de expresión únicamente en el hígado y solo cuando ambos *zeitgeber*s están presentes.

- **Conocer el efecto de una disrupción temporal sobre diferentes osciladores en el carpín**

Una inversión de 12 horas del horario de alimentación provoca un avance de 3-5 horas en las acrofases de los ritmos de los genes reloj y de los receptores nucleares en el oscilador hipotalámico, y un desfase de 12 horas en el reloj hepático. Por su parte, en el tejido interrenal se provoca un gran desajuste entre los genes reloj, asociado a la pérdida del ritmo de cortisol circulante.

CONCLUSIONES

1. La presencia en el carpín de todos los componentes del sistema de las NAEs sugiere que estos derivados lipídicos actúan como moléculas bioactivas en los peces, pudiendo intervenir en la regulación de diferentes funciones fisiológicas.

2. La alimentación es un importante regulador de las NAEs en los peces, como lo demuestran las importantes variaciones en los niveles gastrointestinales de OEA, PEA y SEA, en respuesta a los ciclos diarios de alimentación y ayuno. El rápido aumento postprandial observado en tejidos gastrointestinales, pero no en tejidos cerebrales, sugiere que estas NAEs podrían estar actuando como señales periféricas de saciedad a corto plazo.

3. La PEA presenta un efecto anorético y además reduce el peso corporal, efectos que podrían estar mediados por un aumento de la leptina hepática y una disminución del NPY hipotalámico. La acusada reducción de la actividad anticipatoria a la alimentación apoya un efecto de la PEA no solo en la etapa consumatoria del comportamiento alimentario, sino también en etapas previas de alerta y localización de la comida.

4. Las NAEs regulan el metabolismo hepático en los peces, generando un efecto lipogénico inducido por OEA y PEA, junto con una reducción del potencial lipolítico por la OEA. Además, ambas NAEs reducen el potencial gluconeogénico y aumentan el glucogenolítico. Dichos efectos podrían estar mediados por el aumento de la expresión hepática de *bmal1a* inducido por ambas NAEs.

5. Se ha comprobado que el hipotálamo se comporta como un oscilador sincronizado por la luz (LEO), mientras que el hígado actúa como un oscilador sincronizado por la comida (FEO) en el carpín. Por su parte, la glándula interrenal parece ser más sensible al ciclo luz-oscuridad, sugiriendo que se comporta más como un LEO.

6. Los ritmos diarios de NAEs así como de su receptor PPARα en tejidos gastrointestinales del carpín asociados al momento de la ingesta, sugieren que estos derivados lipídicos pueden señalizar la hora de la comida, actuando como entradas o *inputs* de los FEOs.

7. La expresión rítmica de *rev-erbα* se encuentra ligada a la existencia de ritmos en los genes reloj, estando controlada por el ciclo luz-oscuridad en el hipotálamo y por la alimentación en el hígado. Esto sugiere que en el carpín *rev-erbα* es un gen controlado por el reloj (CCG).

8. La existencia de ritmos diarios en la expresión de *rev-erbβ*, *rorα* y *pparα* solamente en el hígado sugiere que su ritmicidad es más importante para el metabolismo hepático que para otras funciones.

9. El desajuste entre los *zeitgeber*s ciclos luz-oscuridad y alimentación-ayuno afecta al oscilador del tejido interrenal en mayor medida que a los osciladores hipotalámico y hepático. Todo ello apoya que una alteración de la homeostasis temporal es un agente estresante para los peces.

SUMMARY

Role of N-acylethanolamines and the circadian system in the energy homeostasis in teleost fish

INTRODUCTION

Food intake is a multifactorial process driven by a great number of environmental and physiological factors; whose regulation is highly conserved throughout the evolution from teleosts fish to mammals. Thus, in all vertebrates, both central nervous system and a wide variety of peripheral organs work in a coordinated manner to establish optimal regulation of feeding.

A large number of neuroendocrine signals are currently involved in the regulation of energy homeostasis in fish, such as neuropeptides, hormones, and monoamines. However, little is known about molecules of a different nature, such as the lipid derivatives N-acylethanolamines (NAEs), which have also been suggested to be candidates to act as a link between the metabolism and the circadian system in mammals, via their receptor PPARα.

Therefore, the general objective of this Doctoral Thesis is the study of the role of NAEs in the energy homeostasis of fish and to investigate the relationship between the circadian system and this energy homeostasis, using the cyprinid *Carassius auratus* as a model.

OBJECTIVES AND RESULTS

- **Characterize key components of the NAEs system in fish**

It has been demonstrated the presence in the goldfish of all components of the NAEs' system: OEA, PEA, and SEA, their precursors, their synthesis (NAPE-PLD) and degradation

(FAAH) enzymes, and their receptor (PPARα) in different brain (hypothalamus and telencephalon) and gastrointestinal tissues (intestinal bulb, anterior intestine, and liver).

- **Determine the role of NAEs in the regulation of food intake in fish**

Feeding induces an increase in the gastrointestinal levels, but not central ones, of OEA, PEA, and SEA. In addition, PEA reduces food intake, body weight, and locomotor activity, as well as an increase in the gene expression of hepatic *leptin* and a decrease in expression of hypothalamic *npy*.

- **Study the possible effects of OEA and PEA on energy homeostasis and interaction with the circadian system**

Both OEA and PEA cause an increase in lipogenic enzyme (ACLY and FAS, respectively) activity, and a reduction in lipolytic enzyme (CPT-1) activity by OEA. Both NAEs also reduce the PEPCK enzyme and increase GPase. Furthermore, both NAEs modulate the expression of some clock genes in the liver, highlighting an increase in the expression of *bmal1α*. Levels of OEA, PEA, and SEA, as well as the expression of *pparα*, in liver and intestine of goldfish present daily rhythms associated with the time of feeding.

- **Identify whether scheduled feeding and/or light-dark cycle determine the synchronization of oscillator in fish**

Both in hypothalamus and liver, clock genes show daily rhythms of mRNA expression. Such rhythms in the hypothalamus are lost in the absence of light-dark cycle, while they are not affected by changes in scheduled feeding. In the liver, clock-gene rhythms are maintained even in constant darkness, adjusting to the feeding schedule.

- **Check for daily rhythms of the nuclear receptors PPAR, ROR, and REVERB, and determine which *zeitgeber*s drive such rhythms**

The rhythmic expression of *rev-erbα* is controlled by the light-dark cycle in the hypothalamus and by the feeding schedule in the liver. On the other hand, *rev-erbβ*, *rorα*, and *pparα* only present daily rhythms of expression in the liver when both zeitgebers are present.

- **Know the effect of a temporary disruption on different oscillators in goldfish**

A 12-h shift in the scheduled feeding causes a 3-5 h advance in the acrophases of rhythms of clock genes and nuclear receptors in the hypothalamic oscillator, and a 12-h shift in the hepatic clock. On the other hand, there is a great mismatch between the clock genes in the interrenal gland, associated with the loss of circulating cortisol rhythm.

CONCLUSIONS

1. The presence in the goldfish of all components of the NAEs' system suggests that these lipid derivatives act as bioactive molecules in fish and may participate in the regulation of different physiological functions.

2. Feeding is an important regulator of NAEs in fish, as demonstrated by significant variations in gastrointestinal levels of OEA, PEA, and SEA, in response to daily feeding-fasting cycles. The rapid postprandial increase observed in gastrointestinal tissues, but not in brain ones, suggests that these NAEs may be acting as short-term peripheral satiety signals.

3. PEA has an anorectic effect and also reduces body weight, effects that could be mediated by an increase in hepatic leptin and a decrease in hypothalamic NPY. The sharp reduction in anticipatory feeding activity supports an effect of PEA not only in the feeding-behavior consummatory stage, but also in previous stages of awareness and location of food.

4. NAEs regulate hepatic metabolism in fish, producing a lipogenic effect induced by OEA and PEA, as well as a reduction of lipolytic potential only by OEA. In addition, the gluconeogenic potential is reduced and the glycogenolytic one is increased by both NAEs. These effects could be mediated by the increase in *bmal1a* expression induced by both NAEs in the liver.

5. It has been proven that the hypothalamus behaves like a light-entrainable oscillator (LEO), while the liver acts as food-entrainable oscillator (FEO) in goldfish. On the other hand, the interrenal gland seems to be more sensitive to the light-dark cycle, suggesting that it behaves more like a LEO.

6. The feeding-dependent daily rhythms of NAEs and their receptor PPARα in gastrointestinal tissues of goldfish suggest that these lipid derivatives can signal the mealtime, behaving as inputs for FEOs.

7. The rhythmic expression of *rev-erbα* is linked to the presence of clock-gene rhythms, being controlled by the light-dark cycle in the hypothalamus and by feeding in the liver. This confirms that *rev-erbα* is a clock-controlled gene (CCG) in goldfish.

8. The existence of daily rhythms in the expression of *rev-erbβ*, *rorα*, and *pparα* only in the liver suggests that their rhythmicity is more relevant to hepatic metabolism than to other functions.

9. The mismatch between both *zeitgebers* light-dark and feeding-fasting cycles affects the oscillator of the interrenal gland in a greater magnitude than the hypothalamic and hepatic oscillators. All this supports the fact that an alteration of the temporal homeostasis is a stressor for fish.

INTRODUCCIÓN

INTRODUCCIÓN

1 REGULACIÓN DE LA INGESTA EN PECES

La ingestión de alimentos es un proceso multifactorial muy complejo en el que intervienen una gran cantidad de elementos tanto ambientales como fisiológicos, cuya regulación se encuentra muy conservada a lo largo de la evolución, desde peces teleósteos hasta mamíferos. Así en todos los vertebrados, tanto el sistema nervioso central (SNC, el encéfalo en general pero con el hipotálamo como el principal centro regulador) como una gran cantidad de órganos periféricos (tales como el tracto gastrointestinal, el hígado, el páncreas o el tejido adiposo) funcionan de forma coordinada para establecer una perfecta regulación de la ingesta. Esta regulación se produce gracias a la acción de bucles positivos y negativos que actúan en distintas localizaciones y a distintos tiempos (Langhans, 1999), pudiéndose diferenciar tres niveles de regulación de la ingesta (Delgado *et al.*, 2017):

- A corto plazo: intervienen los factores que influyen sobre el tamaño de una sola toma de alimentos.
- A medio plazo: intervienen los factores que operan a lo largo de unos pocos días.
- A largo plazo: intervienen los factores que reflejan el balance energético del animal a lo largo de semanas, meses e incluso años.

A pesar del alto grado de conservación en la regulación de la ingesta entre los peces y los mamíferos, la respuesta específica no es idéntica y en los peces no está perfectamente caracterizada la diferenciación de los tres niveles de regulación anteriormente mencionados (Soengas *et al.*, 2018). El enorme número de especies de peces que existen, la gran heterogeneidad de hábitats en los que viven estos animales, la diversidad de hábitos

alimenticios (herbívoros, carnívoros y omnívoros) con las consecuentes modificaciones anatómicas y fisiológicas del tracto gastrointestinal, o las duplicaciones del genoma que ha sufrido la Clase *Actinopterygii* a lo largo de la evolución, pueden ser algunos de los factores responsables de las características específicas de la regulación de la ingesta en los peces (Hoskins y Volkoff, 2012; Soengas *et al.*, 2018).

A modo de resumen, la **Figura 1** muestra los principales factores reguladores de la ingesta que podemos agrupar en señales **neuroendocrinas** involucradas en la regulación **homeostática** y **hedónica**, factores o información **metabólica** y señales ambientales o información **circadiana**.

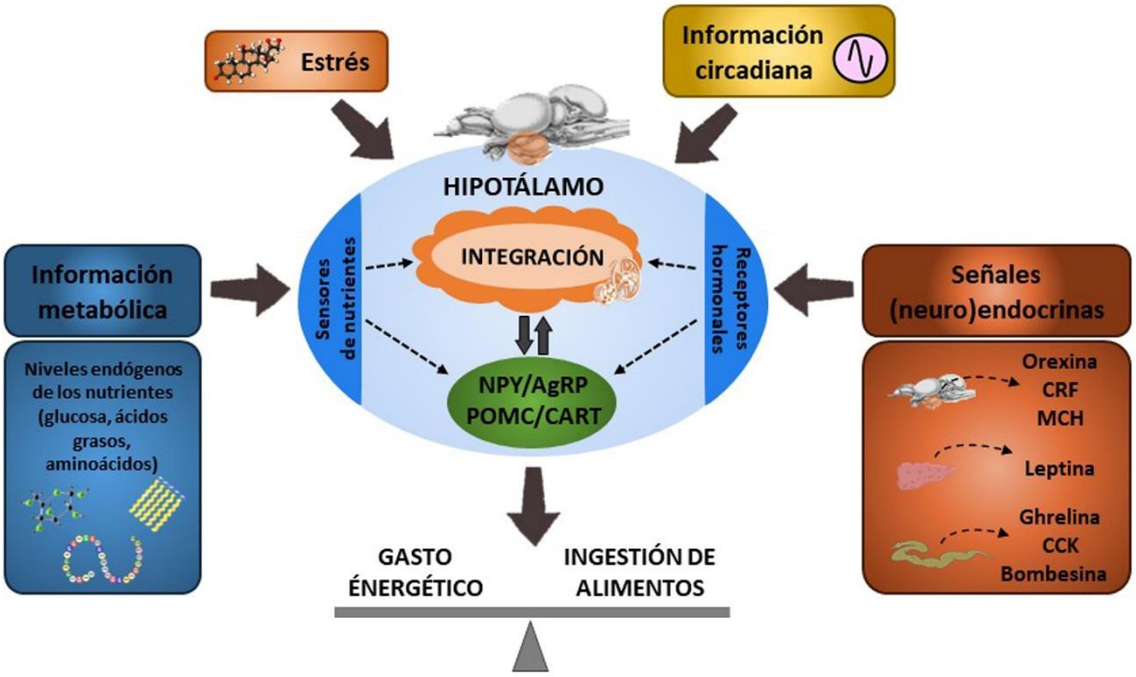


Figura 1. Representación esquemática de la regulación de la ingesta. En la figura se muestran los principales elementos involucrados en el control hipotalámico y periférico de la homeostasis energética en los peces. AgRP, péptido relacionado con Agouti; CART, tránscrito regulado por anfetamina y cocaína; CCK, colecistocinina; CRF, factor de liberación de corticotropina; MCH, hormona concentradora de melanina; POMC, proopiomelanocortina; NPY, neuropéptido Y. Modificada de Delgado *et al.* (2017).

1.1 Regulación homeostática

El control homeostático del apetito es un proceso de vital importancia para los organismos ya que posibilita el correcto mantenimiento de los depósitos de energía en el cuerpo de los animales. En todos los vertebrados, incluidos los peces, esta regulación homeostática de la ingesta está regida por la integración de una serie de factores tanto centrales como periféricos que se pueden clasificar en **orexigénicos** (estimulan la ingesta) y **anorexigénicos o anoréticos** (inhiben la ingesta), y que para su estudio podemos clasificar

como señales hipotalámicas y gastrointestinales, en función de sus principales sitios de acción (Volkoff 2016; Delgado *et al.* 2017; Rønnestad *et al.* 2017; Soengas *et al.* 2018). A continuación se describe brevemente la información de algunas de las señales neuroendocrinas más importantes involucradas en la regulación homeostática de la ingesta en los teleósteos.

1.1.1 Señales hipotalámicas

El hipotálamo es la principal región cerebral responsable de la regulación de la ingesta tanto en peces como en mamíferos (Delgado *et al.*, 2017; Soengas *et al.*, 2018). Esta región se encuentra en la parte ventral del encéfalo y está compuesta por una intrincada red de circuitos orexigénicos y anorexigénicos, gracias a los cuales se integran todas las señales procedentes tanto de otras regiones cerebrales como de la periferia. En el hipotálamo de los peces destacan el área dorsal del núcleo periventricular (NPeVd) y el hipotálamo lateral (HTL), regiones que equivalen al núcleo arqueado de los mamíferos (Jeong *et al.*, 2018).

El **neuropéptido Y (NPY)** es un neuropéptido de 36 aminoácidos (aa) que presenta una amplia distribución por todo el SNC, siendo uno de los más abundantes en el cerebro, y también en tejidos periféricos como el tracto gastrointestinal (Cerdá-Reverter y Larhammar, 2000; Narnaware y Peter, 2002; Kehoe y Volkoff, 2007; MacDonald y Volkoff, 2009a, b; Matsuda *et al.*, 2012). El principal sitio de liberación de NPY es en una población de neuronas localizadas en el NPeVd y el HTL (Cerdá-Reverter y Peter, 2003; Opazo *et al.*, 2019). Es uno de los neuropéptidos con mayor grado de conservación a lo largo de la filogenia de los vertebrados (Blomqvist *et al.*, 1992) y se considera la señal **orexigénica** más potente (López-Patiño *et al.*, 1999; Matsuda *et al.*, 2012; Bertucci *et al.*, 2019). Una inyección intracerebroventricular (ICV) causa una marcada estimulación de la ingesta (López-Patiño *et al.* 1999; Narnaware *et al.* 2000; Silverstein y Plisetskaya 2000), efecto que parece ser mediado por receptores específicos Y_1 (López-Patiño *et al.*, 1999; de Pedro *et al.*, 2000). Además, sus niveles cerebrales presentan variaciones periprandiales (Narnaware *et al.*, 2000; Vera *et al.*, 2007), con incrementos en condiciones de ayuno (Narnaware *et al.*, 2000; Narnaware y Peter, 2001). Las acciones orexigénicas del NPY parecen estar relacionadas con la modulación de otros reguladores del apetito (Volkoff *et al.*, 2009), como por ejemplo los orexigénicos orexina, hormona concentradora de melanina (MCH), galanina y ghrelina (Volkoff y Peter, 2000; Miura *et al.*, 2006; Matsuda *et al.*, 2007) o los anoréticos factor liberador de corticotropina (CRF), tránscrito inducido por la cocaína y la anfetamina (CART) y leptina (Volkoff y Peter, 2000; Volkoff *et al.*, 2003; Bernier *et al.*, 2004).

El **péptido relacionado con Agouti (AgRP)** es un neuropéptido de 109 aa que se produce en el SCN en la población de neuronas que coexpresan NPY dentro de los núcleos hipotalámicos NPeVd y HTL (Cerdá-Reverter y Peter, 2003; Opazo *et al.*, 2019). Al igual que el NPY, este neuropéptido posee un papel **orexigénico** en los peces, que funciona como un antagonista endógeno de los receptores de melanocortinas MC4R, activados por la hormona estimulante de melanocitos (α-MSH; Zhang *et al.* 2012; Sohn 2015; Dalmolin *et al.* 2015; Bertucci *et al.* 2019).

Las **orexinas (OX) A y B** (también llamadas *hipocretinas 1* y *2*) son péptidos de 33 y 28 aa, generados a partir de la proteólisis de un único precursor denominado prepro-orexina (pOX). Presentan una amplia distribución por todo el organismo, aunque los mayores niveles se localizan en el hipotálamo (Kaslin *et al.*, 2004; Xu y Volkoff, 2007; Yan *et al.*, 2011). Presentan un papel **orexigénico**, demostrado por la estimulación de la ingesta que se observa tras un tratamiento ICV con cualquiera de las dos OX (Volkoff *et al.*, 1999; Nakamachi *et al.*, 2006; Yokobori *et al.*, 2011), así como por un aumento en los niveles de expresión de pOX tras un protocolo de ayuno en el cerebro del carpín (*Carassius auratus*; Nakamachi *et al.*, 2006; Abbott y Volkoff, 2011) y del pez ciego mexicano de las cuevas (*Astyanax fasciatus mexicanus*; Wall y Volkoff 2013). Al igual que los anteriores neuropéptidos, las OX parecen ejercer su efecto orexigénico mediante la interacción con otros reguladores del apetito, tales como el NPY, la ghrelina, la leptina o la hormona liberadora de tirotropina (Volkoff *et al.*, 2003; Miura *et al.*, 2007; Abbott y Volkoff, 2011; Yan *et al.*, 2011; Nisembaum *et al.*, 2014).

El **sistema de las melanocortinas** está formado por un conjunto de péptidos que derivan de un precursor común que es la **proopiomelanocortina (POMC)**, el cual posee 194 aa y sufre modificaciones postraduccionales específicas de tejido, pudiendo rendir distintas formas de la MSH (α-, β- y γ-MSH), hormona adrenocorticotropa (ACTH) y otros péptidos como el péptido opioide β-endorfina (Cerdá-Reverter *et al.*, 2003; Metz *et al.*, 2006; Sobrino Crespo *et al.*, 2014; Volkoff, 2016). Una de las poblaciones neuronales más importantes productoras de POMC se encuentra en los núcleos hipotalámicos NPeVd y HTL, que además coexpresan CART. Todo el sistema de las melanocortinas se postula como un conjunto de señales **anoréticas** debido a que tratamientos ICV con α-MSH o con el agonista de sus receptores MC4R genera una inhibición de la ingesta en el carpín (Cerdá-Reverter *et al.*, 2003) y en la trucha arcoíris (*Oncorhynchus mykiss*; Schjolden *et al.* 2009). Además, los niveles de expresión de POMC aumentaron inmediatamente después de la ingesta en el diencéfalo del medaka (*Oryzias latipes*; Chisada *et al.* 2014) y el hipotálamo de la trucha arcoíris (Gong y Björnsson, 2014).

El **tránscrito regulado por cocaína y anfetamina (CART)** es un neuropéptido que se distribuye por todo el organismo, aunque su mayor expresión se colocaliza con la POMC en las neuronas hipotalámicas del NPeVd y HTL. En los peces teleósteos presenta una cantidad de isoformas muy variable dependiendo de la especie, habiéndose identificado dos tránscritos (CART I, de 117 aa; CART II, de 120 aa) en el carpín (Volkoff, 2016; Bertucci *et al.*, 2019). Es un neuropéptido **anorético** que provoca una marcada reducción de la ingesta tras su administración ICV en el carpín (Volkoff, 2016); mientras que sus niveles hipotalámicos disminuyen en situaciones de ayuno y aumentan tras la ingestión de alimentos en distintas especies de teleósteos (Volkoff, 2016; Zhou *et al.*, 2019). El posible mecanismo que subyace a este efecto anorético es a través de un efecto sinérgico con la leptina y de un efecto antagónico sobre el NPY y la OX-A (Volkoff y Peter, 2000).

Además de las señales neuroendocrinas descritas anteriormente, existen otras muchas que también intervienen en la regulación central de la ingesta en los peces. Así, entre las orexigénicas destacan la MCH, la galanina y la apelina. Por su parte, entre las anoréticas cabría señalar al CRF, nesfatina-1, al péptido relacionado con el gen de la calcitonina (CGRP), al polipéptido activador de la adenilato ciclasa (PACAP) y a la urotensina I (de Pedro *et al.*, 1998b; Bernier y Peter, 2001; Volkoff *et al.*, 2009; Kerbel y Unniappan, 2012; Volkoff, 2016; Bertucci *et al.*, 2019).

1.1.2 Señales gastrointestinales

La regulación de la ingesta implica también la acción de señales periféricas procedentes tanto del tracto digestivo como de otros órganos, como el hígado, el páncreas o el tejido adiposo (Woods, 2004; Cummings y Overduin, 2007; Sam *et al.*, 2012). Dentro de estas señales se pueden hacer dos grandes divisiones según el marco de acción que presenten, de tal forma que se dividen en señales periféricas a corto plazo y a largo plazo.

a) Señales periféricas a corto plazo

El principal órgano periférico que secreta una gran cantidad de señales que intervienen en la regulación de la ingesta a corto plazo es el **tracto gastrointestinal**, el cual es considerado uno de los mayores órganos endocrinos (Capasso y Izzo, 2008), produciendo señales tanto orexigénicas (ghrelina) como anoréticas (colecistocinina, bombesina, péptido liberador de gastrina, amilina, péptido YY o péptido similar al glucagón tipo 1), describiendo a continuación algunas de las que actualmente se dispone de más información en los peces.

La **ghrelina (GHR)** destaca por ser el único péptido gastrointestinal que presenta propiedades **orexigénicas** potentes tanto en peces (Kaiya *et al.*, 2008; Kang *et al.*, 2011;

Jönsson, 2013) como en mamíferos (Cummings, 2006; De Vriese y Delporte, 2008; Castañeda *et al.*, 2010). Esta hormona presenta una enorme variabilidad estructural dependiendo de las especies, teniendo una longitud de 12-25 aa en los peces (Bertucci *et al.*, 2019; Kitazawa y Kaiya, 2019), con una secuencia bastante conservada a lo largo de la filogenia (Kaiya *et al.*, 2008). Esta hormona se produce principalmente en el estómago o en el bulbo intestinal (en aquellos peces teleósteos que carecen de un estómago definido) y presenta variaciones periprandiales con mayores niveles previos al momento de la ingesta y una reducción posterior tanto de la hormona a nivel plasmático como de su tránscrito en el bulbo intestinal del carpín (Blanco *et al.*, 2016). Los mecanismos a través los cuales la GHR ejerce su papel orexigénico parecen implicar la activación del NPY hipotalámico, actuando a través de las vías aferentes sensoriales primarias del nervio vago que conectan con los centros de alimentación en el cerebro (Matsuda *et al.*, 2006; Miura *et al.*, 2006).

La **colecistocinina (CCK)** es una hormona **anorética** que se libera como su precursor (preproCCK) de 123 aa en el carpín y que contiene la secuencia octapeptídica (CCK-8S) bioactiva cercana al extremo C-terminal, la cual se encuentra altamente conservada a lo largo de la filogenia (Rønnestad *et al.*, 2017). La administración tanto central como periférica del CCK-8S provoca una reducción de la ingesta tanto en el carpín (Himick y Peter, 1994b) como en el pez gato americano (*Ictalurus punctatus*; Silverstein y Plisetskaya 2000). Sus niveles de expresión se ven modificados con los ciclos ayuno-alimentación, de tal forma que se reducen tanto en el cerebro como en el intestino después de un protocolo de ayuno (Murashita *et al.*, 2006; Ji *et al.*, 2015), mientras que aumentan en el intestino en respuesta a la alimentación. Su papel anorético parece estar mediado en parte por la inhibición del vaciado gástrico (Tinoco *et al.*, 2015), así como también por la sinergia con señales de adiposidad a largo plazo como la leptina o la insulina (Volkoff *et al.*, 2003; Morton *et al.*, 2006).

La **bombesina (BBS)** y el **péptido liberador de gastrina (GRP)** son péptidos homólogos liberados fundamentalmente desde el tracto gastrointestinal que presentan funciones **anoréticas** en diferentes especies de peces (Himick y Peter, 1994a; Xu y Volkoff, 2009; White *et al.*, 2016; Rønnestad *et al.*, 2017). Además, los niveles de expresión de *grp* en el tracto gastrointestinal disminuyen con el ayuno tanto en el bacalao del Atlántico (*Gadus morhua*; Xu y Volkoff 2009) como en el pez cebra (*Danio rerio*; Koven y Schulte 2012), aumentando tras la realimentación. Según Canosa y colaboradores (2005), la acción anorética de estos péptidos se lleva a cabo a través de la reducción en la expresión intestinal de la *ghr*.

b) Señales periféricas a largo plazo

La **leptina** es una hormona secretada principalmente por el hígado en los peces que presenta múltiples formas, siendo la principal forma la leptina A, y probablemente la implicada en la regulación de la ingesta (Gorissen y Flik, 2014; Londraville *et al.*, 2014). En los peces, se ha observado tanto un aumento postprandial en los niveles de expresión de leptina en el hígado, el intestino y el cerebro tras la ingesta, así como un efecto **anorético** tras su administración en diversas especies de teleósteos (Volkoff, 2016), sugiriendo un posible papel de esta hormona como señal de saciedad a corto plazo. Dicha acción anorética parece estar mediada por otros reguladores de la ingesta, como NPY/AgRP y POMC/CART, de manera similar a como ocurre en los mamíferos (van de Pol *et al.*, 2017). Sin embargo, en los peces no está tan claro su conocido papel lipostático demostrado en los mamíferos, encontrando resultados contradictorios al respecto (Gorissen y Flik, 2014; Soengas *et al.*, 2018). En este sentido, hay que tener en cuenta que más allá de la señalización del estado nutricional, la leptina en los peces parece presentar un papel fisiológico más amplio (van de Pol *et al.*, 2017).

La **insulina** es una hormona secretada principalmente por las células β pancreáticas cuyo papel como señal adipostática a largo plazo no está tampoco claro en los peces (van de Pol *et al.*, 2017). Su papel en la periferia como hormona anabólica es más evidente, existiendo una mayor controversia en las funciones de la insulina a nivel central. Por ejemplo, algunos autores han encontrado un efecto anorético tras la administración ICV de insulina en trucha arcoíris (Soengas y Aldegunde, 2004), mientras que en otros estudios no se han encontrado modificaciones significativas de la ingesta en el pez gato americano (Silverstein y Plisetskaya, 2000).

1.2 Regulación hedónica

La regulación neuroendocrina hedónica o también denominada motivacional está basada en los sistemas de recompensa cerebrales que se activan fundamentalmente en respuesta a la ingestión de alimentos con una alta palatabilidad, los cuales ejercen una sensación de placer y recompensa. Aunque en los mamíferos se han involucrado al sistema opioide endógeno, al sistema endocannabinoide, así como al sistema dopaminérgico mesolímbico como componentes de la regulación hedónica de la ingesta (Nogueiras *et al.*, 2012; Lau *et al.*, 2017), en los peces estos aspectos se encuentran muy poco estudiados.

Los **péptidos opioides**, como las endorfinas, están implicados en la regulación de la ingesta en el carpín, desarrollando una acción **orexigénica**, como en mamíferos, ya que se ha demostrado que la administración ICV de β-endorfina incrementa la ingesta a tiempos cortos,

efecto que es revertido tanto por el antagonista general naloxona como por el antagonista de los receptores opioidérgicos μ (de Pedro *et al.*, 1995, 1996). En relación al **sistema endocannabinoide**, en los peces solamente se conoce el papel **orexigénico** de la anandamida (AEA) tras su administración oral en la dorada (*Sparus aurata*; Piccinetti *et al.* 2010) o tras su administración IP en el carpín (Valenti *et al.*, 2005). Los pocos estudios que hay en peces sugieren que el **sistema mesolímbico dopaminérgico** puede estar involucrado en la regulación motivacional de la alimentación. En concreto, se ha demostrado que una administración oral de L-DOPA inhibe la ingesta en la lubina (*Dicentrarchus labrax*; Leal *et al.* 2013), al igual que la administración ICV de agonistas de los receptores dopaminérgicos también inhiben la ingesta en el carpín (de Pedro *et al.*, 1998a); resultados que concuerdan con los efectos hipotalámicos de la señalización dopaminérgica en el pez cebra (Yamamoto *et al.*, 2011).

1.3 Regulación metabólica

La ingestión de alimentos va a provocar variaciones en los niveles de los nutrientes, de tal forma que se activan quimiorreceptores en la parte proximal del tracto gastrointestinal, produciendo señales que viajan a través de fibras aferentes del nervio vago hasta distintas regiones del cerebro, donde se integran para provocar la inhibición de la ingesta (Soengas, 2014). Por otro lado, los nutrientes que llegan al hígado a través del sistema porta-hepático también van a desencadenar señales aferentes a través de las mismas fibras nerviosas. De esta forma, los peces son capaces de regular la cantidad de comida ingerida gracias a la información de los niveles de glucosa, aminoácidos y ácidos grasos, que es detectada a través de los denominados **sistemas sensores** del cerebro, principalmente en el hipotálamo (Soengas *et al.*, 2018).

Teniendo en cuenta que los lípidos son una fuente importante de energía en los peces (Tocher, 2003; Polakof *et al.*, 2010), es fácil imaginar que tanto los **sistemas sensores de ácidos grasos** como el metabolismo lipídico van a desarrollar un importante papel en la regulación de la homeostasis energética. Por un lado, se ha demostrado una reducción de la ingesta tras una alimentación con dietas ricas en lípidos, en peces con altos niveles plasmáticos de ácidos grasos o con grandes acúmulos de lípidos, así como tras tratamientos con ácido oleico u octanoleico (Soengas *et al.*, 2018). Los principales mecanismos hipotalámicos sensores de ácidos grasos conocidos en los mamíferos también parecen estar presentes en los peces, con algunas particularidades (Soengas *et al.*, 2018). Así, un incremento de los niveles plasmáticos de ácidos grasos (tanto de cadena larga como media en los peces) produce a nivel hipotalámico un aumento de los niveles de la enzima *malonil-Coenzima A*, inhibiendo la enzima *carnitina palmitoiltransferasa tipo 1* (*CPT-1*), encargada de introducir los ácidos grasos

a la mitocondria para su oxidación. Además, también se ha descrito un incremento en la capacidad del traslocador de ácidos grasos FAT/CD36 y un descenso en la expresión de las enzimas *ATP-citrato liasa* (*ACLY*) y *citrato sintasa*.

A pesar de que el metabolismo de la glucosa en los peces es más limitado que en los mamíferos, parece que los **mecanismos glucosensores** también operan de manera comparable**.** Así, una alimentación con dietas ricas en carbohidratos o unas condiciones hiperglucemiantes provocan una reducción de la ingesta, y viceversa (Soengas *et al.*, 2018). En los últimos años, diversos estudios en peces han apoyado la presencia en el hipotálamo de los principales mecanismos glucosensores conocidos en los mamíferos. Uno de estos mecanismos es el mediado por la enzima *glucoquinasa* (*GK*; Conde-Sieira y Soengas 2017), por la cual la glucosa introducida en la neurona hipotalámica es fosforilada por la *GK* y posteriormente metabolizada en la glucolisis incrementándose la cantidad de ATP intracelular. Este aumento de la ratio ATP/ADP induce cambios en los canales de K^+ regulados por ATP (K^+_{ATP}), lo que provoca una despolarización de la membrana y la consecuente entrada de Ca^{2+} al interior celular. Otros mecanismos glucosensores presentes en los peces están relacionados con el receptor X hepático (LXR) y los receptores del gusto para el sabor dulce (Otero-Rodiño *et al.*, 2015, 2016; Balasubramanian *et al.*, 2016).

Por último, los **mecanismos sensores de aminoácidos** son los más inexplorados tanto en mamíferos como en peces. Aun así, en los últimos años se han desarrollado diferentes estudios en los que se ha visto que dietas con altos niveles de aa o proteínas inhiben la ingesta, y viceversa (Soengas *et al.*, 2018). Además, Comesaña y colaboradores (2018) han determinado que inyecciones ICV del aa leucina reducen la ingesta en la trucha arcoíris, lo que parece estar asociado con la presencia de sistemas sensores de aminoácidos en el hipotálamo.

Tal y como se ha indicado anteriormente, tanto el metabolismo de lípidos como de glúcidos son muy importantes para el mantenimiento de la homeostasis energética en los peces. En la **Figura 2** se muestran las principales rutas implicadas en el metabolismo de la glucosa y de los lípidos en los peces teleósteos, en donde se remarcan las principales enzimas implicadas.

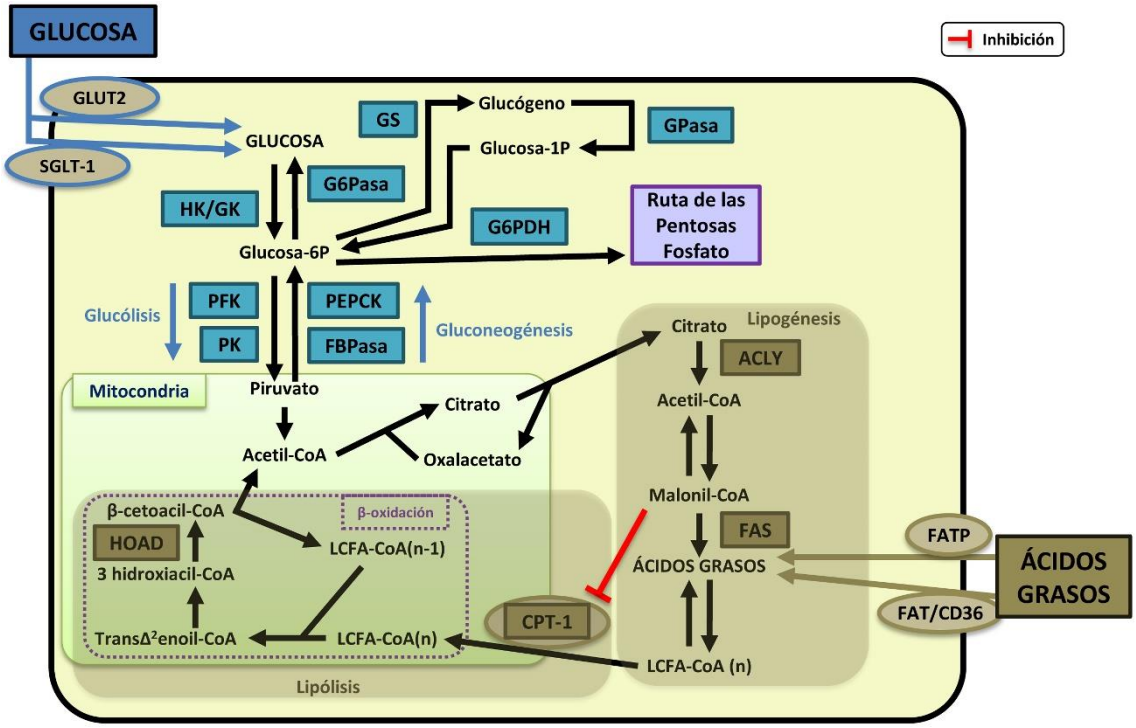


Figura 2. Principales rutas metabólicas de glúcidos y lípidos. ACLY, ATP citrato liasa; CPT-1, carnitina palmitoiltransferasa tipo 1; FAS, ácido graso sintasa; FAT/CD36, translocasa de ácidos grasos acoplada al clúster de diferenciación 36; FATP, proteína transportadora de ácidos grasos; FBPasa, fructosa-1,6-bifosfatasa; G6Pasa, glucosa-6 fosfatasa; G6PDH, glucosa-6-fosfato deshidrogenasa; GK, glucoquinasa; GLUT2, transportador bidireccional de glucosa; GPasa, glucógeno fosforilasa; GS, glucógeno sintasa; HK, hexoquinasa; HOAD, L-3-hidroxiacil-CoA deshidrogenasa; LCFA-CoA, ácidos grasos de cadena larga unidos a Coenzima A; PEPCK, fosfoenolpiruvato carboxiquinasa; PFK, fosfofructoquinasa; PK, piruvato quinasa; SGLT-1, traslocador acoplado sodio-glucosa.

2 N–ACILETANOLAMINAS

2.1 Estructura de los ligandos

Las **N–aciletanolaminas (NAEs)**, también llamadas amidas de ácidos grasos o aciletanolamidas, son una familia de moléculas endógenas de naturaleza lipídica que se encuentran presentes en todos los seres vivos, desde animales, plantas e incluso células procariotas (Hansen y Diep, 2009; Hansen y Vana, 2018). Estos derivados lipídicos se encuentran formados por la unión, mediante un enlace de tipo amida, de un ácido graso y una etanolamina. Dependiendo del tipo de ácido graso que intervenga en la formación de la NAE, esta tomará el nombre determinado por dicho lípido. De esta manera, se pueden diferenciar una gran cantidad de NAEs, algunas de las cuales se muestran en la **Figura 3**.

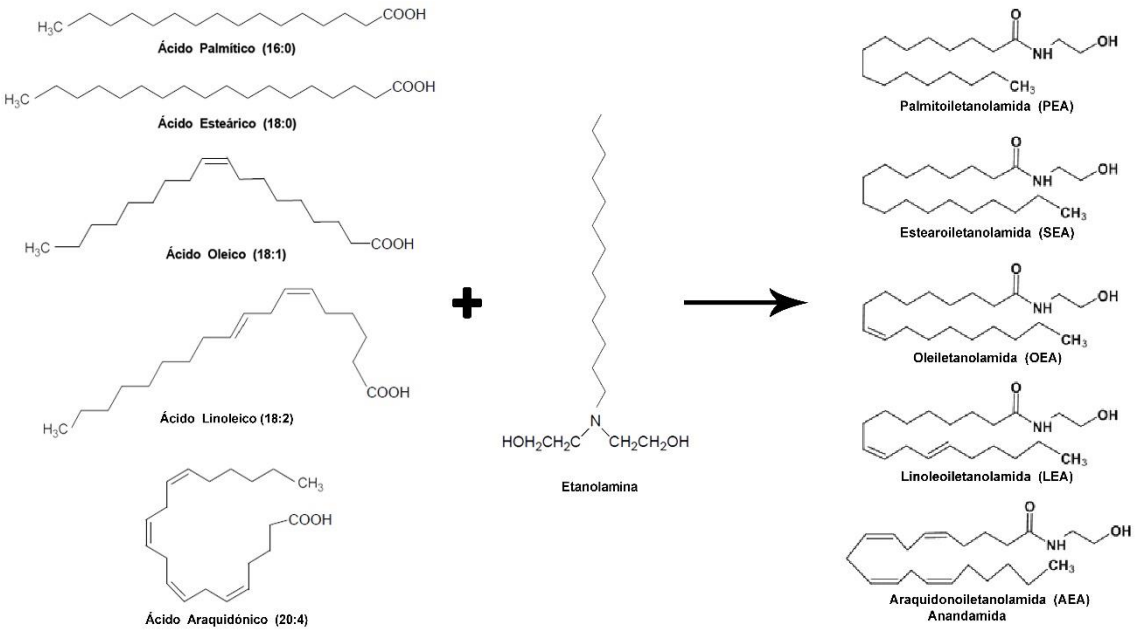


Figura 3. Estructura de diferentes NAEs, indicando el ácido graso que interviene en la formación de cada una de ellas y la longitud de la cadena (número de carbonos) y la saturación de la molécula (número de dobles enlaces que se forman).

En los siguientes apartados nos centraremos fundamentalmente en la OEA, la PEA y la SEA, debido a que son las NAEs objeto de estudio de la presente Tesis Doctoral.

2.2 Síntesis, distribución y degradación

La **biosíntesis** celular de las NAEs se realiza mediante un proceso que se denomina "a demanda", estando los niveles endógenos de estas moléculas regulados principalmente por las propias enzimas de síntesis y degradación. En los mamíferos, se ha descrito ampliamente el proceso de síntesis de las NAEs (Okamoto *et al.*, 2004; Borrelli e Izzo, 2009; Tsuboi *et al.*, 2013; Ueda *et al.*, 2013; Rahman *et al.*, 2014; Inoue *et al.*, 2017), existiendo cuatro rutas compuestas por dos grandes pasos, el primero de los cuales es común a todas ellas (**Figura 4**). Así, en primer lugar se produce la formación del precursor específico de cada una de las NAEs denominado N-acilfosfatidiletanolamina (NAPE, del inglés *N-acylphosphatidylethanolamines*) a partir de dos compuestos localizados en las membranas celulares que son las fosfatidiletanolaminas (PE, del inglés *phosphatidylethanolamines*) y los glicerofosfolípidos (GPL, del inglés *glycerophospholipids*), los cuales contienen a los ácidos grasos correspondientes, gracias a la acción de la enzima *N-aciltransferasa* (*NAT*) en un proceso dependiente de Ca^{2+}. Posteriormente, la conversión de NAPE a NAE se puede producir por cuatro vías distintas. La llamada vía clásica se ha considerado la predominante y la más directa, teniendo lugar simplemente mediante la hidrólisis de cada NAPE liberándose su respectiva NAE, reacción catalizada por una enzima *fosfolipasa D* (*PLD*, del inglés *phospholipase D*) específica para las NAPEs que se encuentra unida a la membrana y se denomina *NAPE–PLD*.

Una segunda ruta se lleva a cabo mediante una reacción doble en cadena formándose en primer lugar una lisoNAPE principalmente por la acción de la enzima *fosfolipasa secretora A_2* (s*PLA_2,* del inglés *secretory phospholipase A_2*) y a continuación entra en acción una PLD específica del sustrato denominada *lisoPLD*. En tercer lugar, y como alternativa a la opción anterior, se encuentra una reacción triple en cadena mediante la cual a partir de la NAPE se forma la lisoNAPE por la acción de la *PLA_2* o de la *α/β hidrolasa 4* (*ABH4*), posteriormente se

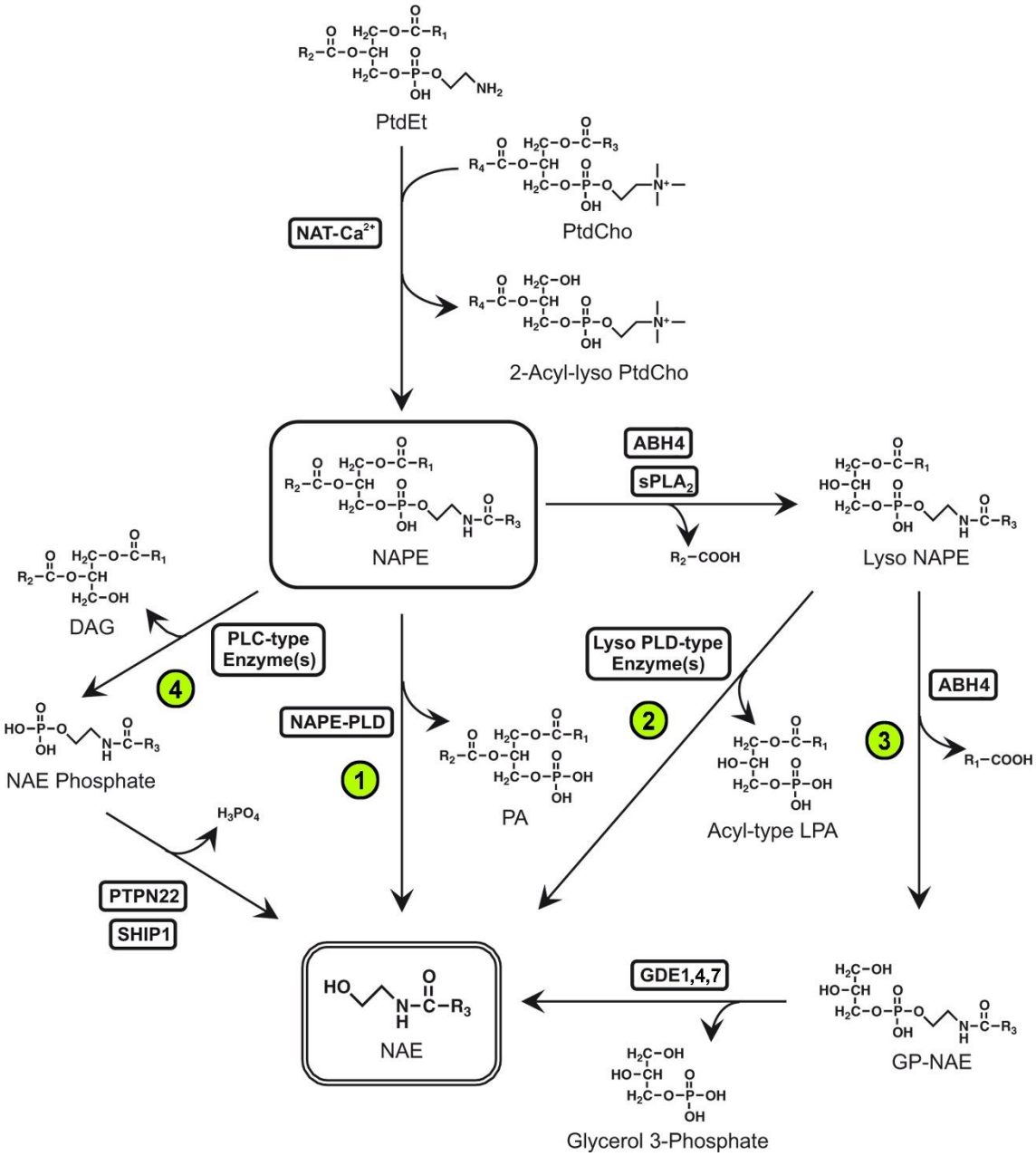


Figura 4. Biosíntesis de las N-aciletanolaminas. Representación esquemática de las cuatro posibles rutas de formación de NAEs a partir de fosfatidiletanolaminas (PtdEt) y glicerofosfolípidos de la membrana (PtdCho, fosfatidilcolina). ABH4, α/β hidrolasa 4; DAG, diacilglicerol; GDE, glicerofosfodiesterasa; GP-NAE, *sn*-glicerol-3-fosfato-NAE; LPA, ácido lisofosfatídico; NAPE, N-acilfosfatidiletanolamina; NAT, N-aciltransferasa; PA, ácido fosfatídico; PLC, fosfolipasa C; PLD, fosfolipasa D; PTPN22, proteína tirosina fosfatasa intracelular N22; SHIP1, inositol-5-fosfatasa; $sPLA_2$, fosfolipasa secretora A_2. Modificada de Rahman *et al.* (2014).

genera *sn*–glicero–3–fosfo–NAE (GP–NAE) por acción de la enzima *ABH4* nuevamente y, por último, las enzimas *glicerofosfodiesterasas* (*GDE,* del inglés *glycerophosphodiesterase*) *1, 4* ó *7* transforman la GP–NAE en la correspondiente NAE libre. La cuarta vía se corresponde con una reacción doble por la cual mediante la *fosfolipasa C* (*PLC,* del inglés *phospholipase C*) la NAPE se convierte en NAE fosfato para que posteriormente se transforme en la correspondiente NAE mediante la acción de diferentes enzimas *fosfatasas*.

En los mamíferos, las NAEs presentan una amplia **distribución**, variando sus niveles dependiendo de los tejidos y las especies (Kleberg *et al.*, 2014). Así, se han descrito cantidades detectables de OEA, PEA, SEA, LEA y AEA, entre otras NAEs, en todo el tracto gastrointestinal (glándulas salivares, estómago, duodeno, yeyuno, íleon y colon), tejido adiposo, hígado, riñón, páncreas, bazo, pulmón, músculo, tejido adiposo, corazón y testículos. Además de encontrarse periféricamente, en el cerebro se localizan en el tálamo, hipotálamo, corteza cerebral o córtex, estriado, hipocampo, cerebelo y tronco encefálico. Incluso, se han detectado niveles de OEA y PEA tanto en el plasma como en el líquido cerebroespinal, aunque estos niveles circulantes son mucho más bajos que en los tejidos (Fu *et al.*, 2007; Hansen, 2013; Kleberg *et al.*, 2014; Bowen *et al.*, 2017). Cuantitativamente, en la mayoría de los tejidos de rata y ratón, las NAEs con los niveles más altos son OEA, PEA y SEA, a excepción del yeyuno en el que la NAE más abundante es la LEA (Artmann *et al.*, 2008; Hansen, 2013; Kleberg *et al.*, 2014; Rahman *et al.*, 2014; Tsuboi *et al.*, 2018). En cuanto a los vertebrados no mamíferos, solamente se han estudiado los niveles de NAEs en tres especies: el reptil pitón de Birmania (*Python molurus*) y los peces cebra y carpín. En la pitón de Birmania, se detectaron niveles de OEA, PEA y LEA en el estómago, el intestino anterior (máximas cantidades) y el colon, con una distribución parecida a la descrita en los mamíferos, siendo OEA y PEA las más representativas. En los peces teleósteos estudiados, ambos de la familia *Cyprinidae*, se han reportado cantidades de OEA y PEA en cerebro, hígado y gónadas del pez cebra (Martella *et al.*, 2016; Forner-Piquer *et al.*, 2018), y de OEA en hipotálamo, telencéfalo, cerebelo, tronco encefálico, bulbo intestinal, intestino anterior, hígado y músculo del carpín (Tinoco *et al.*, 2014a).

Los niveles tisulares de NAEs se encuentran principalmente regulados por el estado nutricional del animal. Así, los niveles intestinales de OEA descienden debido a protocolos de ayuno y se restablecen tras la realimentación en mamíferos (Rodríguez de Fonseca *et al.*, 2001; Piomelli, 2013), reptiles (Astarita *et al.*, 2006) y peces (Tinoco *et al.*, 2014a). Cabe destacar la rapidez con la que ocurre todo este proceso (10-30 min, dependiendo de las especies), lo que hace considerar a la OEA como una señal rápida de saciedad. En los mamíferos y los reptiles se sabe que la principal movilización de la OEA en el intestino viene determinada por la

incorporación del ácido oleico de la dieta a los enterocitos, lo que provoca una estimulación de la *NAPE-PLD* y una inhibición de la *FAAH* (enzima de degradación), favoreciéndose la producción de OEA (Fu *et al.*, 2007; Piomelli, 2013). En los peces este mecanismo podría ser muy parecido, ya que administraciones tanto centrales como periféricas del ácido oleico tienen un efecto anorético en la trucha arcoíris, a través de mecanismos relaciones con el metabolismo lipídico probablemente mediados por los sistemas sensores de ácidos grasos (Librán-Pérez *et al.*, 2012, 2014). De las otras dos NAEs principales, PEA y SEA, se han descrito diferentes resultados. Así, según Petersen y colaboradores (2006) la movilización de PEA generada tras la alimentación se produce de forma similar a la de OEA en el intestino de rata, sin existir modificaciones en los niveles de SEA. En cambio, Fu *et al.* (2007) no encontraron ningún cambio en los niveles de PEA y SEA en el duodeno y el yeyuno de rata después de un protocolo de ayuno.

La degradación o **catabolismo** de las NAEs ocurre gracias a una hidrolisis enzimática por la cual se producen el ácido graso libre correspondiente y una etanolamina. Este proceso se puede llevar a cabo por dos enzimas distintas, cada una de las cuales presenta distinta afinidad por las diferentes NAEs. Así, la principal enzima de degradación es la denominada *amidohidrolasa de ácidos grasos* (*FAAH,* del inglés *fatty acid amide hydrolase*), localizada en las membranas del retículo endoplásmico y de la mitocondria. Su mayor actividad enzimática se encuentra en el hígado, el intestino delgado y el cerebro (Hansen, 2010; Ueda *et al.*, 2013; Kleberg *et al.*, 2014). En humanos y otros mamíferos placentarios se describió otra isoforma de esta enzima a la que denominaron *FAAH-2*, pasándose a llamar la primera *FAAH-1* (Wei *et al.*, 2006; Piomelli, 2013). Esta segunda isoforma también realiza el catabolismo de las NAEs, preferiblemente de la OEA (Bowen *et al.*, 2017). La ruta catabólica alternativa de las NAEs se realiza a través de la denominada *amidasa ácida hidrolizante de NAEs* (*NAAA,* del inglés *N-acylethanolamine acid amide hydrolase*). Esta enzima hidroliza preferiblemente la PEA, presentando una menor afinidad por la OEA y se localiza principalmente en los lisosomas de los macrófagos del bazo, el intestino delgado y el timo, teniendo la máxima actividad enzimática y expresión génica en los macrófagos alveolares de los pulmones (Ueda *et al.*, 2013; Bottemanne *et al.*, 2018).

Todo el sistema de enzimas de síntesis y degradación descrito en los mamíferos parece estar muy conservado en los peces teleósteos. Así, se han descrito genes ortólogos de las enzimas de síntesis *NAPE-PLD* y de degradación principal *FAAH-1*, así como de las rutas alternativas de síntesis *Abdh4*, *lysoPLD* y *GDE1* en pez cebra (GenBank: NM_200243.1, McPartland *et al.*, 2007) y en el salmón del Atlántico (*Salmo salar*; GenBank: BT072143.1). Por

otro lado, de algunos de los genes se han generado parálogos debido a los fenómenos de duplicación genómica experimentada en los teleósteos a lo largo de la evolución. Tal es el caso de la enzima de degradación *FAAH-2* existiendo en el pez cebra *FAAH-2a* y *FAAH-2b*. Sin embargo, parece ser que la enzima de degradación *NAAA* se ha perdido en el pez cebra (McPartland *et al.*, 2007), no existiendo datos en otros peces hasta el momento.

2.3 Receptores

En los mamíferos se han descrito diferentes tipos de receptores a los cuales se unen las N–aciletanolaminas con mayor o menor afinidad (**Tabla 1**).

Tabla 1. Principales receptores diana de las NAEs.

Receptores diana	NAEs
PPARα	OEA, PEA, SEA
PPARβ/δ	¿OEA?
GPR119	OEA, PEA
GPR55	OEA, PEA
TRPV1	OEA, PEA, SEA
Canales iónicos de Na^+, K^+, Ca^{2+}	¿OEA, PEA y SEA?

Para el significado de las abreviaturas véase el texto.

De entre todos ellos destacan los **receptores activados por proliferadores de peroxisomas** (**PPARs**, del inglés *peroxisome proliferator-activated receptors*), dentro de los cuales existen tres subtipos: PPARα, PPARγ y PPARβ/δ (**Figura 5**). El receptor principal al cual se van a unir las NAEs es **PPARα**, con una alta afinidad tanto por la OEA como la PEA. La activación de este receptor provoca su conexión con el receptor de retinoide X formando un heterodímero que se une a los sitios de respuesta a PPAR (PPRE, del inglés *PPAR response elements*), situados en los promotores de sus genes diana en el ADN, para regular su transcripción (Bowen *et al.*, 2017). Estudios *in vitro* han demostrado que la OEA también puede activar PPARβ/δ, pero no PPARγ. No obstante, estudios *in vivo* con agonistas para cada isoforma de PPAR demostraron que los

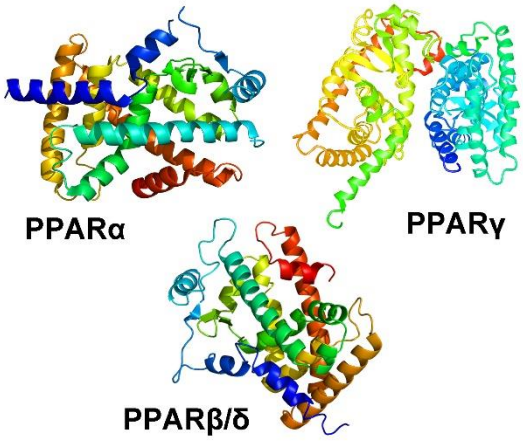


Figura 5. Estructura tridimensional de los tres subtipos de PPAR.

efectos sobre la regulación de la ingesta que ejerce la OEA cuando activa PPARα solamente se replican con los propios agonistas de este receptor (Wy-14643 y GW7647), mientras que los agonistas de las otras dos isoformas (GW501516 y ciglitazona, respectivamente) no alteraron la ingesta (Fu *et al.*, 2003; Bowen *et al.*, 2017; Hansen y Vana, 2018).

Las NAEs también pueden activar otros tipos de receptores como son los **receptores huérfanos acoplados a proteínas G** (**GPRs**, del inglés *G-protein-coupled receptors*; **Figuras 6a y 6b**) y el **receptor de potencial transitorio activado por vanilloides tipo 1** (**TRPV1**, del inglés *transient receptor potential cation channel subfamily V member 1*; **Figura 6c**). Dentro de los GPRs, tanto el **GPR119** como el **GPR55** se activan por OEA y por PEA. La distribución del GPR119 es principalmente en el páncreas y en células intestinales, mientras que el GPR55 presenta sus mayores niveles de expresión en las glándulas adrenales y el tracto gastrointestinal, con una menor representación en el SNC (Ryberg *et al.* 2007; Godlewski *et al.* 2009; Lauffer *et al.* 2009; Hansen 2010; Kleberg *et al.* 2014). Por su parte, el TRPV1 es un receptor sensible a la capsaicina que se encuentra principalmente localizado en las neuronas aferentes sensoriales del nervio vago, pudiendo ser activado por las distintas NAEs (Bowen *et al.*, 2017). Por último, también se ha descrito la activación de distintos **canales de iones** (como canales de sodio, de potasio o de calcio) por parte de las NAEs (Kleberg *et al.*, 2014; Hansen y Vana, 2018).

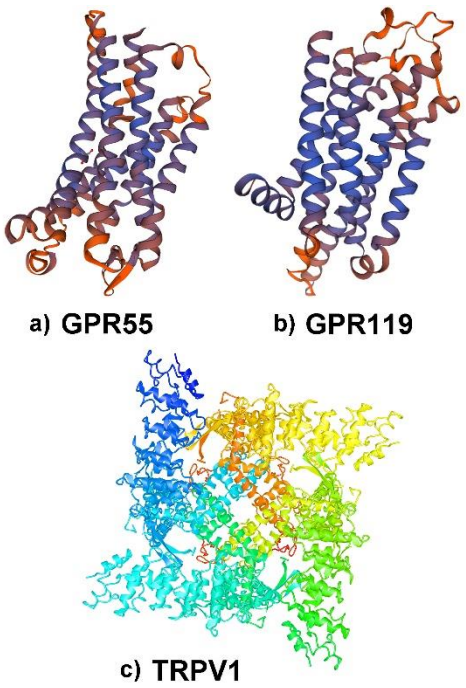


Figura 6. Estructura tridimensional de los dos subtipos de GPR (a y b) y de TRPV1 (c).

En cuanto a los peces, se ha descrito la presencia de las tres isoformas de los PPARs en diferentes especies de teleósteos, con una distribución tisular muy similar a la presente en los mamíferos (Mimeault *et al.*, 2006; Zheng *et al.*, 2013; Carmona-Antoñanzas *et al.*, 2014; Den Broeder *et al.*, 2015; Houston *et al.*, 2017). Por su parte, aunque se ha demostrado la presencia de los otros tres receptores descritos en los mamíferos (GPR119, GPR55 y TRPV1; Fredriksson *et al.* 2003; Zimov y Yazulla 2004; McPartland *et al.* 2007; Gau *et al.* 2013; Krug *et al.* 2018), las posibles implicaciones de estos receptores mediando las acciones de las NAEs están aún por dilucidar en los peces.

2.4 Actividad biológica

Las NAEs presentan una gran actividad biológica en los animales debido a la unión y activación de los distintos receptores descritos anteriormente (**Figura 7**), siendo importantes reguladores periféricos de la homeostasis energética en los vertebrados (Hansen y Vana, 2018). De entre todas las NAEs, la más ampliamente estudiada ha sido la OEA, confirmándose un efecto anorético tras su administración tanto IP como oral en los mamíferos (Piomelli, 2013; Brown *et al.*, 2017; Hansen y Vana, 2018). Los mecanismos subyacentes a esta reducción de la ingesta están mediados por el receptor nuclear PPARα y se producen gracias a la

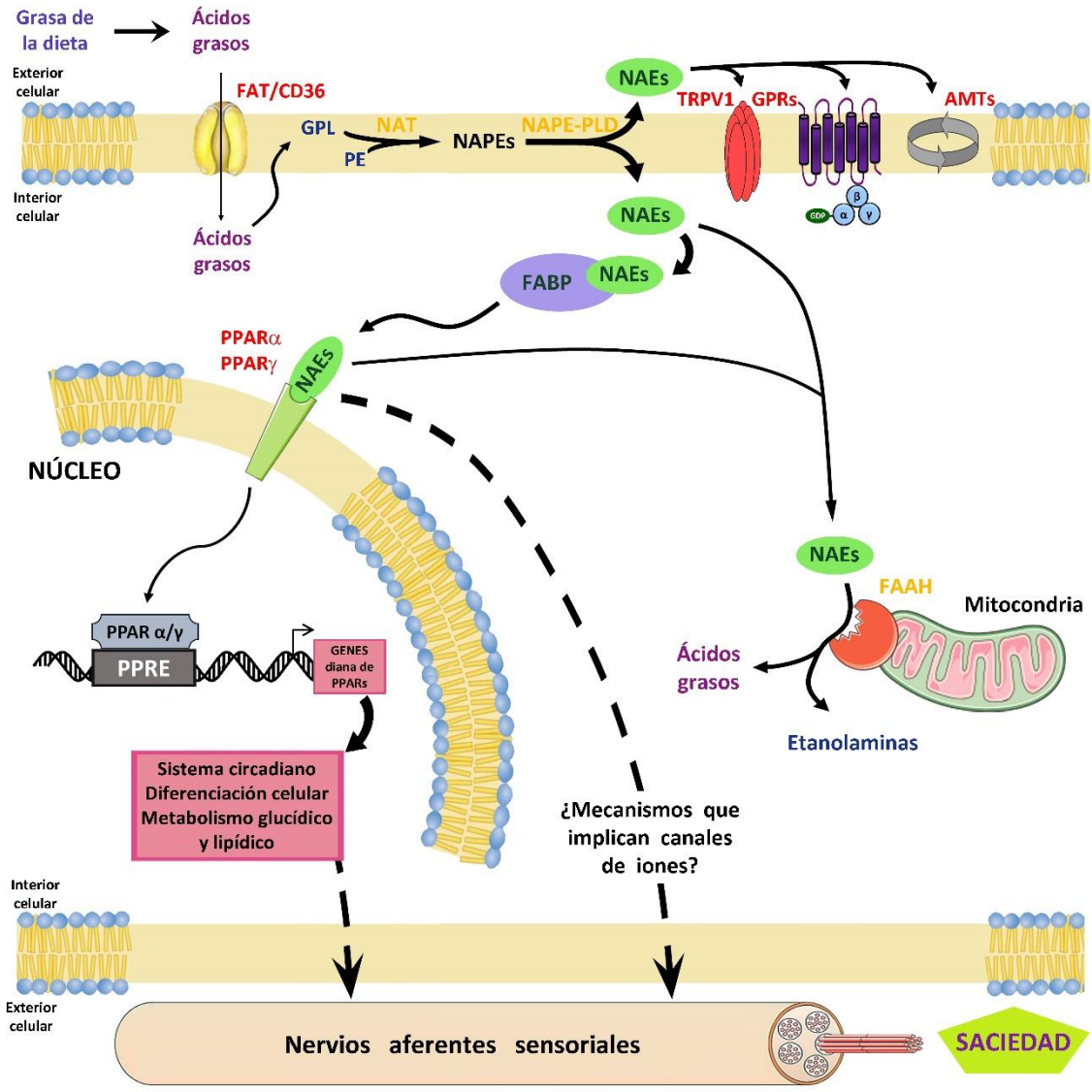


Figura 7. Mecanismo de transmisión de la señal de las NAEs. AMTs, transportadores específicos de aciletanolamidas; FAAH, amidohidrolasa de ácidos grasos; FABP, proteínas de unión a ácidos grasos; FAT/CD36, translocasa de ácidos grasos acoplada al clúster de diferenciación 36; GDP-αβγ, subunidades de la proteína G acopladas a guanosín difosfato; GPL, glicerofosfolípidos; GPRs, receptores huérfanos acoplados a proteínas G; NAPE-PLD, fosfolipasa D específica de NAPEs; NAPEs, N-acilfosfatidiletanolaminas; NAT, N-aciltransferasa; PE, fosfatidiletanolaminas; PPAR, receptores activados por proliferadores de peroxisomas; PPRE, elementos de respuesta a PPAR; TRPV1, receptor de potencial transitorio activado por vanilloides tipo 1.

activación de aferencias sensoriales situadas en la rama intestinal del nervio vago, las cuales viajan hasta el núcleo del tracto solitario (NTS) del tronco encefálico (Rodríguez de Fonseca *et al.*, 2001; Fu *et al.*, 2011; Piomelli, 2013). Del NTS, salen fibras nerviosas que llegan a los núcleos paraventricular (NPV) y supraóptico del hipotálamo activando a las neuronas magnocelulares productoras de oxitocina (Gaetani *et al.*, 2010; Piomelli, 2013; Romano *et al.*, 2013), así como modulando la actividad monoaminérgica y la expresión génica del neuropéptido CART en el NPV (Serrano *et al.*, 2011). Por su parte, la acción de la OEA sobre los neuropéptidos gastrointestinales no está del todo clara. Algunos estudios en ratas muestran que la OEA provoca una disminución en los niveles plasmáticos de ghrelina y de péptido YY (Cani *et al.*, 2004; Serrano *et al.*, 2011), pero en cambio otros indican que la acción anorética de la OEA no es mediada por dichos neuropéptidos (Proulx *et al.*, 2005). En relación con los peces teleósteos, trabajos recientes de nuestro grupo de investigación han descrito un papel de la OEA en la regulación de la ingesta en el carpín de forma similar a la descrita en los mamíferos, demostrando una movilización intestinal de la OEA tras la alimentación y un efecto anorético tras la administración IP de esta NAE (Tinoco *et al.*, 2014a). Esta reducción de la ingesta parece estar mediada por un descenso en la expresión de ghrelina en el bulbo intestinal de estos animales, no encontrándose modificaciones en la expresión génica tanto de la colecistocinina (CCK) intestinal como de la leptina hepática e hipotalámica, al igual que ocurría en los mamíferos (Fu *et al.*, 2005; Proulx *et al.*, 2005). Aunque los estudios sobre el papel de las otras NAEs en la regulación de la ingesta son escasos, parece que también presentan un papel anorético. En concreto, la SEA reduce la ingesta de forma dosis-dependiente tras dos horas de su inyección IP o de su administración oral en el ratón (Terrazzino *et al.*, 2004). En relación con la PEA, su administración tanto aguda como crónica (5 semanas) reduce la ingesta en ratas (Rodríguez de Fonseca *et al.*, 2001; Mattace Raso *et al.*, 2014b).

Además del efecto anorético descrito anteriormente, la OEA presenta acciones sobre el peso corporal y el metabolismo. Así, se ha observado una marcada reducción del peso corporal tanto en rata como en ratón tras un tratamiento con OEA crónico (1 semana; Rodríguez de Fonseca *et al.* 2001; 2-5 semanas; Fu *et al.* 2005; Thabuis *et al.* 2010, 2011). Esta reducción del peso corporal no solamente se atribuye a la reducción en la ingestión de alimento, sino que está relacionada con el metabolismo lipídico promoviendo la lipólisis e inhibiendo la lipogénesis (LoVerme *et al.*, 2005; Matias *et al.*, 2007; Pavón *et al.*, 2010; Thabuis *et al.*, 2011). En cuanto a la acción de las otras dos NAEs sobre el peso corporal, se ha demostrado que un tratamiento crónico (5 semanas) con PEA produce una disminución en el peso corporal en un

modelo de obesidad inducida por ovariectomía en ratas (Mattace Raso *et al.*, 2014b), sin existir datos acerca del posible efecto de la SEA sobre el peso corporal. Tal y como se comentó previamente, tanto la PEA como la SEA presentan efectos anoréticos, los cuales se ligan con modificaciones en el metabolismo lipídico en el mismo sentido que las descritas en relación con la OEA. Así, la PEA provoca un aumento de la lipólisis debido a un aumento en los niveles de expresión de la enzima *CPT-1* en el tejido adiposo (Mattace Raso *et al.*, 2014b), mientras que la SEA genera una reducción de la lipogénesis causada por una caída en la expresión génica de la enzima *estearoil-Coenzima A desaturasa 1* (*SCD1*, del inglés *stearoyl-CoA desaturase 1*) en el hígado (Terrazzino *et al.*, 2004).

Otro de los parámetros que se ve afectado por el tratamiento con las NAEs es la actividad locomotora. En este sentido, una administración aguda de OEA provoca una reducción en la actividad locomotora de las ratas de en torno al 60% (Rodríguez de Fonseca *et al.*, 2001). Esta disminución en el movimiento de los roedores parece ser totalmente independiente de la acción hipofágica producida paralelamente por esta NAE, ya que el tratamiento con capsaicina bloquea específicamente la acción anorética de la OEA sin modificar la locomoción. Datos muy similares se observaron en el carpín, en el que un tratamiento agudo de OEA provocó una disminución de la actividad natatoria de aproximadamente un 35% (Tinoco *et al.*, 2014a). Por su parte, la PEA parece tener efectos distintos dependiendo del modelo experimental utilizado. Así, una inyección aguda con esta NAE generó también una disminución en la actividad locomotora en ratón similar a la observada con la OEA, marcada por una reducción tanto de la distancia total recorrida (descenso ≈ 50%) como del número de posturas erguidas (≈ 75%) en el tablero de agujeros (Zambrana-Infantes *et al.*, 2018). En cambio, cuando se administra la PEA de forma crónica (28 días) después de haber sometido a las ratas a un estrés crónico durante 35 días, los comportamientos exploratorios de los animales, los cuales se habían visto claramente reducidos por el estrés, se recuperan y vuelven a los niveles basales previos al protocolo de estrés, de tal forma que la distancia recorrida y el tiempo total de locomoción aumentan, mientras que los periodos de inmovilidad disminuyen (Li *et al.*, 2019). En cuanto a la SEA no existen datos hasta la fecha que concreten su posible papel en la actividad locomotora.

Por último, en la última década han surgido numerosas investigaciones que describen el papel de las NAEs sobre otras funciones en los mamíferos. Por ejemplo, las tres NAEs, aunque con mayor eficacia la PEA, ejercen una potente acción antiinflamatoria (Dalle Carbonare *et al.*, 2008; Hansen, 2010; Tsuboi *et al.*, 2018). De hecho, los efectos antiinflamatorios de la PEA son tanto centrales (Skaper *et al.*, 2015) como periféricos a nivel intestinal (Borrelli *et al.*, 2015), así

como generando una reducción de la astrogliosis (Holubiec *et al.*, 2018). A nivel cerebral, la PEA juega un papel muy importante como neuroprotector, interviniendo en una gran cantidad de enfermedades del sistema nervioso, tales como el Alzheimer, el Parkinson, la esclerosis múltiple o la epilepsia (Citraro *et al.*, 2013; Mattace Raso *et al.*, 2014a; Petrosino y Di Marzo, 2017; Tsuboi *et al.*, 2018). También se ha descrito la implicación de OEA y PEA en otras funciones como en la consolidación de la memoria (Campolongo *et al.*, 2009; Mazzola *et al.*, 2009), en la modulación de la respuesta al estrés a nivel circulatorio (Hill *et al.*, 2009) o en relación con el sistema circadiano (Murillo-Rodriguez *et al.*, 2006).

3 SISTEMA CIRCADIANO

En el ambiente se producen cambios constantemente debidos a los movimientos de traslación de la Tierra alrededor del Sol, de rotación sobre su propio eje y de rotación de la Luna alrededor de la Tierra. Estas fluctuaciones son cíclicas y generan, respectivamente, la

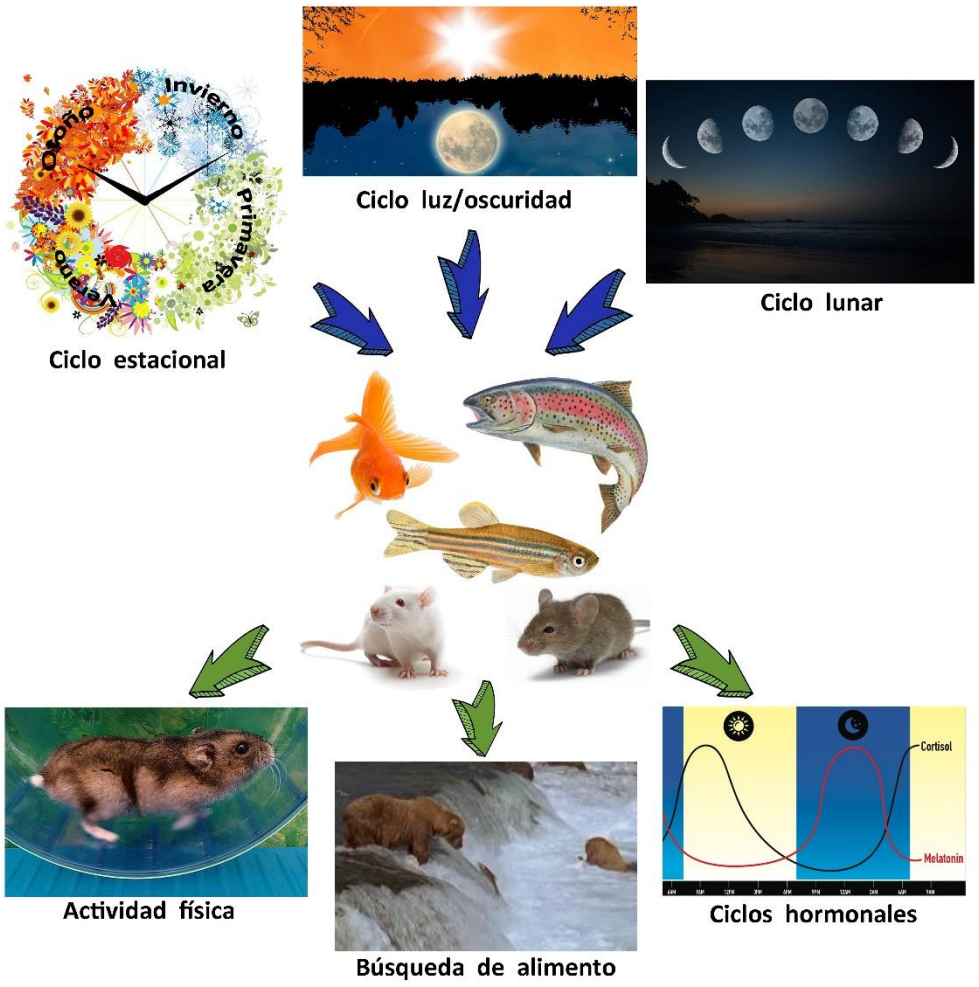


Figura 8. Cambios ambientales cíclicos que generan cambios fisiológicos y comportamentales en los animales.

alternancia de las estaciones del año, de la luz del día y la oscuridad de la noche y los ciclos lunares. Los seres vivos perciben todos estos cambios ambientales adaptando sus procesos vitales para sincronizarse a ellos, pudiendo incluso llegar a anticiparse a dichos cambios (**Figura 8**; Pittendrigh, 1993). La capacidad que tienen los organismos para hacer frente a las fluctuaciones ambientales cíclicas genera una serie de respuestas fisiológicas y/o comportamentales también rítmicas, entre las que se encuentran la temperatura corporal, la secreción hormonal, los ciclos de reproducción, el ciclo sueño–vigilia, los períodos de actividad–reposo, los momentos de alimentación–excreción, etc. (Madrid, 2006; Refinetti, 2006).

La **Cronobiología** (del griego *kronos* – tiempo, *bios* – vida y *logos* – ciencia) es la disciplina de la biología que estudia los ritmos biológicos en los seres vivos, es decir, las variaciones de las funciones biológicas en función del tiempo. Para poder describir estas variaciones de las funciones biológicas de los seres vivos en función del tiempo, en Cronobiología se emplean una serie de parámetros específicos de estos ritmos biológicos que, al ser expresados en unidades numéricas, permiten cuantificarlos (**Figura 9**; Koukkari y Sothern, 2006):

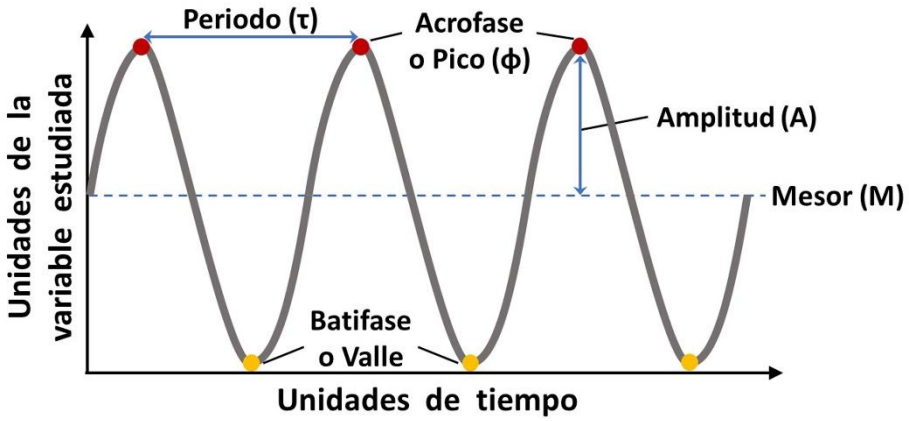


Figura 9. Representación gráfica de los parámetros que caracterizan a los ritmos.

- **Periodo (τ)**: representa el intervalo de tiempo necesario para completar un ciclo. Se expresa en unidades de tiempo. Relacionado con el periodo está otro parámetro que se denomina **Frecuencia (1/τ)** y equivale al número de ciclos por unidad de tiempo.
- **Mesor (M)**: es el valor medio del parámetro (o de la variable) a lo largo de un ciclo, calculado a lo largo de un período completo y ajustado a una función sinusoidal. Se expresa en las unidades de la variable rítmica cuantificada.
- **Fase**: es un punto de referencia temporal de un ritmo. Cuando el ritmo se ajusta a un perfil sinusoidal, las fases más características serían el momento del valor máximo,

denominado **Acrofase** o **Pico (ϕ)**, y el momento del valor mínimo, llamado **Batifase** o **Valle**. La Fase se expresa en unidades de tiempo.

- **Amplitud (A)**: distancia que existe entre el valor medio del ritmo o mesor y el valor máximo alcanzado por la variable en el momento de la acrofase. Se expresa en las unidades de la variable rítmica cuantificada.

En función de la duración de sus periodos, los ritmos biológicos se pueden clasificar según se indica en la **Tabla 2**. De esta forma, por ejemplo, los ritmos con una periodicidad cercana a las 24 horas se denominan circadianos (del latín *circa* – aproximadamente y *diem* – día; Refinetti, 2006).

Tabla 2. Denominación de los ritmos biológicos en función de la duración de su periodo.

Tipo de ritmo	Duración del periodo	Nomenclatura del ritmo	Ejemplo
Ultradianos (< 20 h)	1 hora	Circahoral	Secreción pulsátil de hormonas
	12,4 horas	Circamareal	Actividad de especies costeras
Circadianos (≈ 24 h)	Entre 20 y 28 horas	Circadiano	Sueño–vigilia Temperatura corporal
Infradianos (> 28 h)	28 días	Circalunar	Alimentación en salmónidos Reproducción de animales de zona intermareal
	Entre 10 y 14 meses	Circanual o Estacional	Migración Reproducción

Por otro lado, los ritmos biológicos se pueden clasificar según su origen en exógenos y endógenos. Los ritmos exógenos son aquéllos que se generan como una respuesta pasiva a las variaciones puntuales de un factor ambiental y, una vez que se elimina dicha alteración, desaparece inmediatamente el ritmo. Por su parte, los ritmos endógenos se mantienen invariables en ausencia de un sincronizador o *zeitgeber* externo, lo que se denomina comúnmente como "curso libre" o "*free running*" (Aschoff, 1981). Además, el carácter endógeno de un ritmo biológico implica la existencia de un mecanismo autosostenible denominado **reloj** u **oscilador circadiano** (Pittendrigh, 1993; Mendoza y Challet, 2009), el cual es capaz de modificar su funcionamiento cuando está expuesto a estímulos rítmicos externos (los sincronizadores o *zeitgebers*) hasta tal punto que se igualan los periodos del reloj circadiano con el del estímulo ambiental, proceso denominado **Sincronización** o **Encarrilamiento** (**Figura 10**; Cahill y Besharse 1991, 1993). En cambio, el proceso de **Enmascaramiento** o ***Masking*** ocurre cuando un *zeitgeber* genera un ritmo biológico circadiano

observable sin provocar una sincronización en el reloj, ya que al retirar el estímulo externo no se mantiene la fase entre ambos (Cambras Riu, 2006). En resumen, para que se genere un ritmo biológico endógeno son necesarios los siguientes elementos:

- Vías de entrada (*inputs*): estímulos ambientales que sincronizan el reloj.
- Osciladores circadianos: sistema capaz de generar por sí mismo la oscilación.
- Vías de salida (*outputs*): ritmos biológicos observables.

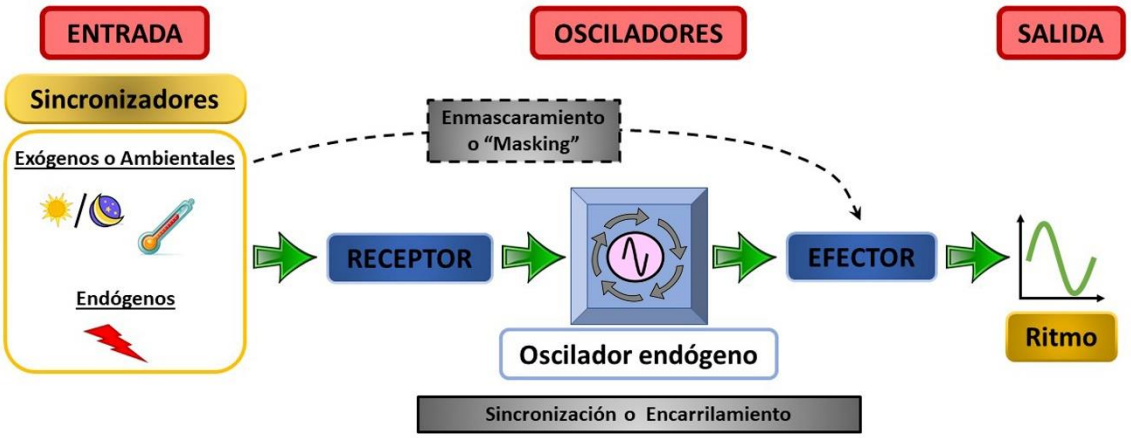


Figura 10. Principales elementos de un sistema circadiano. Los osciladores reciben la información de los sincronizadores o *zeitgebers* a través de los receptores para generar los ritmos correspondientes a través de los efectores oportunos. El enmascaramiento o *Masking* se produce cuando los propios sincronizadores provocan un ritmo en los efectores sin pasar por los osciladores. Modificada de Cambras Riu (2006).

3.1 Base molecular de los relojes endógenos

La maquinaria de los relojes circadianos está muy conservada a lo largo de la evolución (desde organismos unicelulares hasta los vertebrados) y se basa en bucles de retroalimentación entre los procesos de transcripción y traducción de los denominados **genes reloj** y sus productos proteicos, con un periodo aproximado de 24 horas (Panda *et al.*, 2002b; Buhr y Takahashi, 2013). La mitad positiva del bucle principal está conformada por dos genes que actúan como factores de transcripción llamados ***clock*** (del inglés *circadian locomotor output cycles kaput*) y ***bmal1*** (*brain y muscle ARNT-like 1*, también conocido como *arntl*, *aryl-hydrocarbon receptor nuclear translocator-like*). Una vez que salen al citoplasma y se traducen a proteínas, se forma el heterodímero CLOCK:BMAL1 retornando al interior del núcleo, donde promueve la expresión de los genes reloj de la mitad negativa del bucle principal llamados ***per*** (*period*) y ***cry*** (*cryptochrome*). Esta unión se produce gracias a una región básica hélice-lazo-hélice que reconoce las secuencias reguladoras denominadas "cajas E" (E-box; CACGTG) presentes en los promotores de los genes *per* y *cry* (Gekakis *et al.*, 1998; Nakamura *et al.*, 2008). Los transcritos de estos genes salen al citoplasma y se traducen a sus proteínas

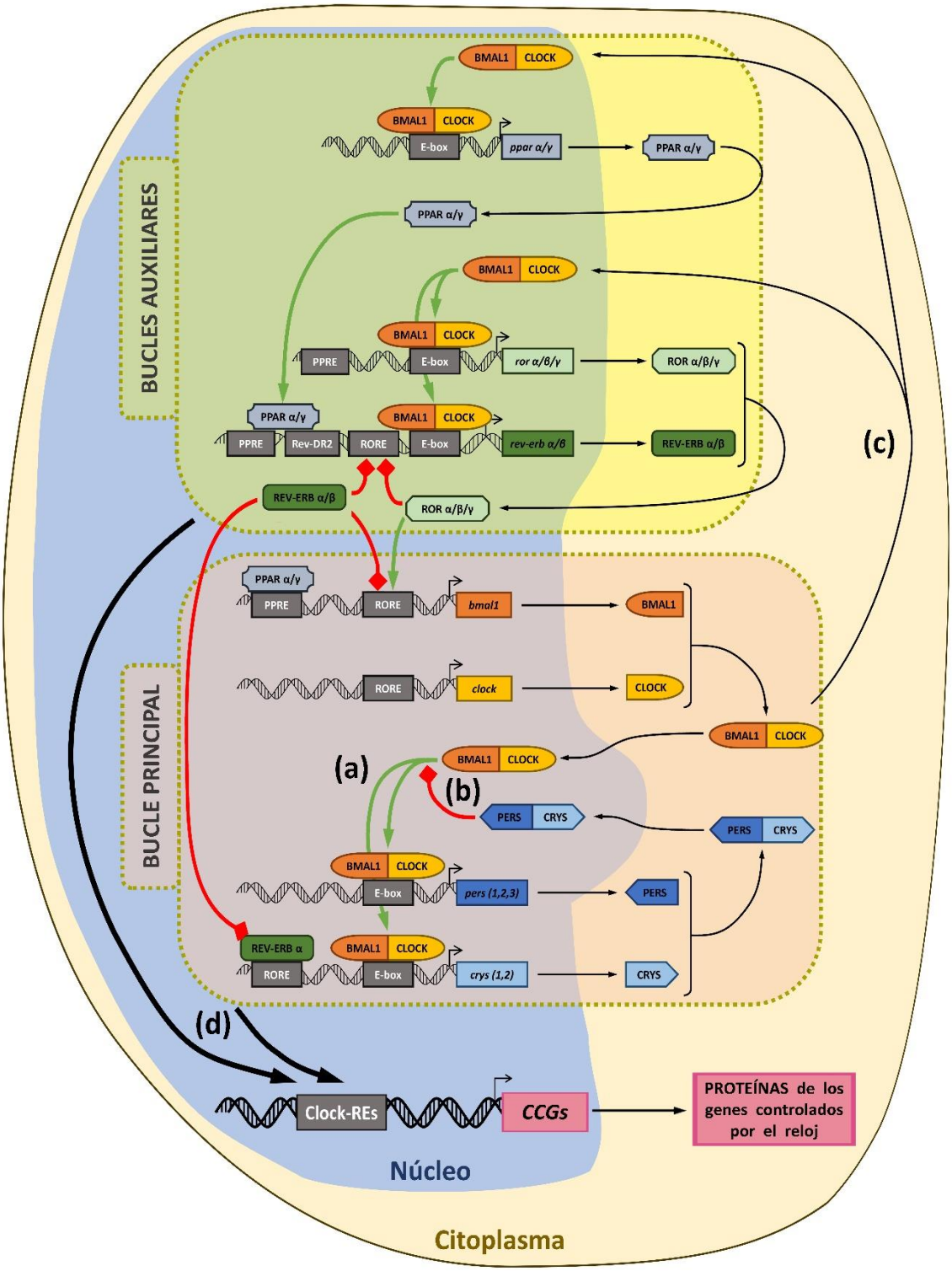


Figura 11. Funcionamiento de los osciladores circadianos en mamíferos. (**a**) El heterodímero CLOCK:BMAL1 favorece la expresión de los genes *per* y *cry*. (**b**) Las proteínas PER y CRY reprimen la transcripción de sus propios genes mediante una inhibición de la acción del heterodímero CLOCK:BMAL1. (**c**) El heterodímero CLOCK:BMAL1 también estimula la expresión de otras proteínas relacionadas, como REV-ERBs, RORs y PPARs. (**d**) Tanto el heterodímero CLOCK:BMAL1 como algunos de los receptores nucleares de los bucles auxiliares controlan la expresión de los CCGs (*clock-controlled genes*). Para una mayor información, consultar el texto. bmal1, *brain y muscle ARNT-like 1*; clock, *circadian locomotor output cycle kaput*; crys, *cryptochromes*; DR2, *direct repeat* separado por 2 pares de bases; pers, *periods*; ppars: *peroxisome proliferator-activated receptors*; PPRE, *peroxisome proliferator response element*; ROR, *retinoic acid related orphan receptor*; RORE, *ROR response elements*.

correspondientes, las cuales sufren una serie de modificaciones postraduccionales, como por ejemplo fosforilaciones de la proteína PER a través de varias caseínas kinasas, acetilaciones, desacetilaciones y ubiquitinaciones (Vanselow y Kramer, 2007; Mehra *et al.*, 2009). Gracias a estos cambios proteicos se unen ambas proteínas formando el heterodímero PER:CRY, el cual se transloca al interior del núcleo reprimiendo la transcripción de sus propios genes mediante la separación de los heterodímeros CLOCK:BMAL1 de las cajas E (Partch *et al.*, 2014; Schibler *et al.*, 2015). Por último, existen una serie de bucles auxiliares que ayudan al mantenimiento de la ritmicidad diaria confiriéndole una mayor robustez a todo el sistema circadiano. Estos bucles auxiliares se encuentran formados principalmente por las proteínas de los receptores nucleares REV-ERBs, RORs y PPARs (Albrecht y Ripperger, 2018), de los que se proporcionará una descripción más completa posteriormente.

De todo el mecanismo descrito anteriormente, el heterodímero CLOCK:BMAL1 va a ser el encargado de controlar las salidas rítmicas del oscilador a través de los denominados genes controlados por el reloj (CCGs; Albrecht y Ripperger 2018). La regulación de estos CCGs se lleva a cabo mediante la unión directa del heterodímero CLOCK:BMAL1 a las cajas E presentes en los promotores de dichos genes, modificando así su transcripción (Hastings *et al.*, 2007; Vatine *et al.*, 2011; Albrecht, 2012). Algunos de estos CCGs codifican para proteínas muy importantes involucradas en funciones biológicas, como por ejemplo enzimas relacionadas con el metabolismo glucídico o la secreción de insulina (Qian y Scheer, 2016; Albrecht y Ripperger, 2018; Peng *et al.*, 2019). La **Figura 11** muestra un resumen del funcionamiento de los osciladores circadianos a través del bucle principal y los bucles auxiliares.

Algunos CCGs forman parte a su vez del bucle auxiliar del oscilador endógeno, tales como los receptores nucleares REV-ERBs, RORs y PPARs (Bozek *et al.*, 2009; Cho H *et al.*, 2012). Así, los receptores nucleares de las familias **REV-ERB** (*reverse strand of protein ERB*, también denominados *NR1D*, *nuclear receptor subfamily 1, member D*; Preitner *et al.*, 2002) y **ROR** (*retinoic acid related orphan receptor*, también denominados *NR1F*, *nuclear receptor subfamily 1, member F*; Sato *et al.*, 2004) generan un bucle de represión y activación de la expresión de los genes *clock* y *bmal1*, respectivamente. Aunque todos los miembros de las dos familias de receptores nucleares (REV-ERBα o NR1D1, REV-ERBβ o NR1D2, RORα o NR1F1, RORβ o NR1F2 y RORγ o NR1F3) intervienen en la regulación de la ritmicidad del sistema circadiano, los dos principales que compiten por unirse a los elementos RORE (*ROR response elements*) presentes en los promotores de los genes *clock* y *bmal1* son el represor **REV-ERBα** y el activador **RORα** (Cho H *et al.*, 2012). También se ha visto que REV-ERBα reprime la expresión de *cry1*, mecanismo que tiene lugar principalmente en el hígado (Pett *et al.*, 2018).

Complementariamente, el heterodímero CLOCK:BMAL1 se une a las cajas E presentes en los promotores de los receptores nucleares REV-ERB y ROR para inducir su transcripción (Schibler *et al.*, 2015), mientras que tanto PER2 como CRY1 reprimen la expresión de *rev-erbα* (Pett *et al.*, 2018). Por otro lado, el promotor del gen *rev-erbα* también contiene un elemento RORE funcional a través del cual se produce la represión del gen por la unión tanto de su propia proteína como por parte de RORα (Delerive *et al.*, 2002; Raspè *et al.*, 2002). Esta autorregulación REV-ERBα-RORα asegura que la expresión de ambos genes se encuentre en antifase mejorando la precisión, solidez y sostenibilidad del reloj circadiano. Por su parte, los receptores nucleares PPARs, específicamente **PPARα** y en menor medida **PPARγ**, intervienen como activadores directos de la transcripción del gen *bmal1* gracias a la formación de heterodímeros con RXR, otro receptor nuclear, para poder unirse a los denominados sitios PPRE (*PPAR response element*) localizados en el promotor de dicho gen (Canaple *et al.*, 2006; Wang *et al.*, 2008; Maradonna y Carnevali, 2018). Además, ambos PPAR también provocan el aumento de la transcripción del gen *rev-erbα* gracias a su unión tanto en forma de heterodímero PPAR/RXR a los sitios PPRE como formando homodímeros PPAR/PPAR a los sitios Rev-DR2 (*Direct Repeat of the AGGTCA motif spaced by 2 nucleotide*) del promotor (Gervois *et al.*, 1999; Laitinen *et al.*, 2005; Canaple *et al.*, 2006; Lecarpentier *et al.*, 2014). En ambos casos, la conexión es recíproca, de tal forma que el heterodímero CLOCK:BMAL1 se une a las cajas E presentes en los promotores de *ppars* y *rev-erbα* provocando un aumento en la transcripción de ambos receptores nucleares (Preitner *et al.*, 2002; Canaple *et al.*, 2006).

Toda la maquinaria molecular y el funcionamiento del sistema circadiano se encuentran altamente conservados a lo largo de la evolución, siendo la principal diferencia el mayor número de copias de todos los genes reloj en los peces teleósteos respecto a los mamíferos debido a los fenómenos de duplicación genómica experimentada en los teleósteos a lo largo de la evolución (Kobayashi *et al.*, 2000; Cahill, 2002; Wang, 2008a, b, 2009). Así, cuando se comparan las cuatro familias de genes reloj que conforman el bucle principal de los osciladores (*per*, *cry*, *clock* y *bmal*) entre el carpín (modelo animal utilizado en la presente Tesis Doctoral) y los mamíferos, podemos observar que la mayoría de los genes de las cuatro familias han sufrido las duplicaciones correspondientes existiendo varias copias de los genes en los teleósteos por cada gen en los mamíferos (**Figura 12**). En ciertos casos, algunas de las copias en determinadas familias de peces se han perdido a lo largo de la evolución (como por ejemplo el caso del gen *per2b* en la Familia *Cyprinidae* o el caso de *bmal1b* en el Superorden *Otophysi*), al igual que se han perdido algunos genes en los mamíferos que se han mantenido en los peces

(como por ejemplo *cry* en la Superclase *Tetrapoda* o el caso de *clock2* en la Infraclase *Placentaria*).

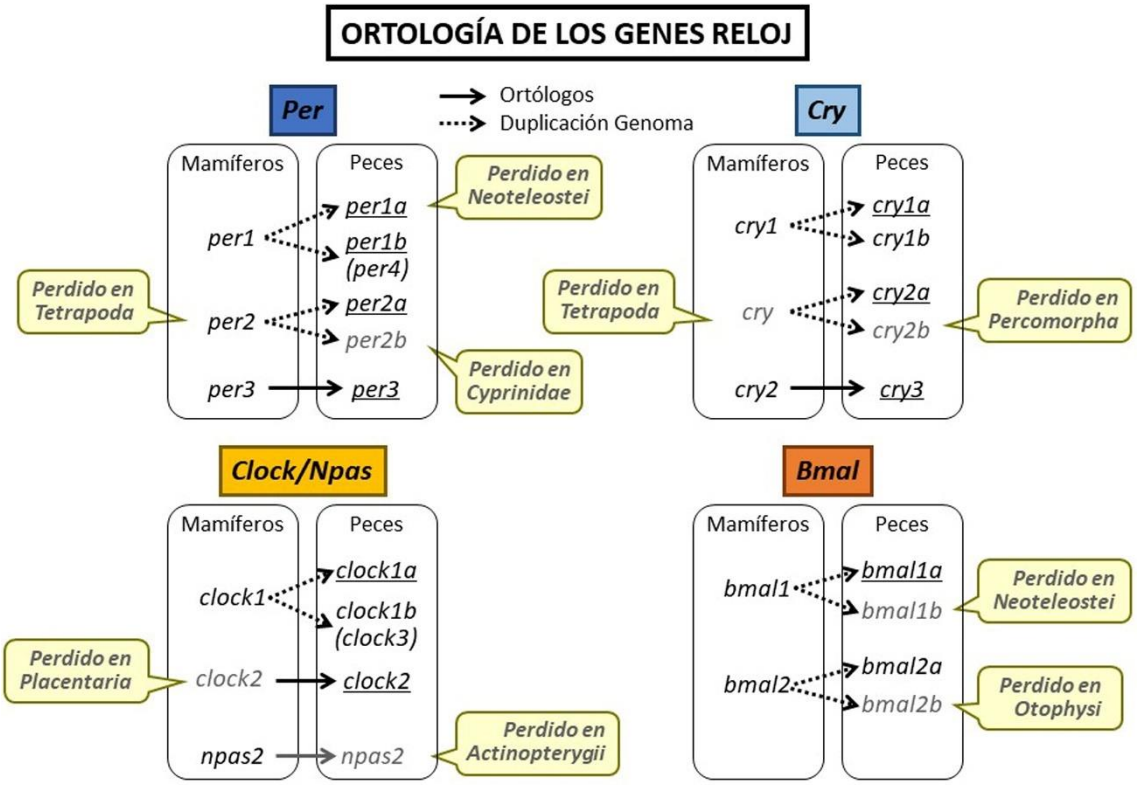


Figura 12. Ortología comparativa de los genes reloj en mamíferos y peces. Las flechas continuas indican la ortología, mientras que las flechas discontinuas indican la duplicación del genoma específico de los teleósteos. Los genes subrayados han sido parcialmente clonados en el carpín, mientras que los genes en gris se han perdido en los grupos indicados en los globos. Los genes entre paréntesis indican nombres alternativos para algunos genes. Los genes en negro y no subrayados no han sido clonados en el carpín, aunque se presume su presencia. Modificada de Sánchez-Bretaño *et al.* (2015a).

3.2 Organización funcional del sistema circadiano

El sistema circadiano de los organismos multicelulares está compuesto por una extensa red de osciladores endógenos distribuidos por todo el cuerpo de los seres vivos que se encuentran complejamente comunicados entre sí (Albrecht, 2012; Bosler *et al.*, 2015; Dibner y Schibler, 2015).

En el caso de los mamíferos, el sistema circadiano presenta una organización jerárquica en la cual existe un reloj maestro que es el **núcleo supraquiasmático del hipotálamo** (SCN, del inglés *suprachiasmatic nuclei*), que controla al resto de relojes endógenos haciendo que todas las células del individuo oscilen de forma sincrónica (**Figura 13**; Albrecht 2012; Partch *et al.* 2014). El SCN es el único reloj molecular que recibe información directa procedente del exterior en forma de luz solar gracias a las conexiones que existen entre la retina y este núcleo, de tal forma que se produce la sincronización entre el reloj interno del SCN y la luz diaria.

Posteriormente, el SCN envía información al resto del cerebro y del cuerpo a través de una combinación de señales neurales, humorales y sistémicas (Buhr y Takahashi, 2013; Partch *et al.*, 2014). Entre los tejidos periféricos en los que se han descrito la existencia de relojes endógenos se encuentran la hipófisis, la retina, el hígado, el corazón, el músculo, el tejido adiposo, el páncreas, el intestino, el riñón, los pulmones, el ovario y la glándula suprarrenal (Balsalobre *et al.*, 2000; Brown *et al.*, 2002; Mühlbauer *et al.*, 2004; Peirson *et al.*, 2006; Zvonic *et al.*, 2006; García-Fernández *et al.*, 2007; Sládek *et al.*, 2007; Girotti *et al.*, 2009; Bass y Takahashi, 2010).

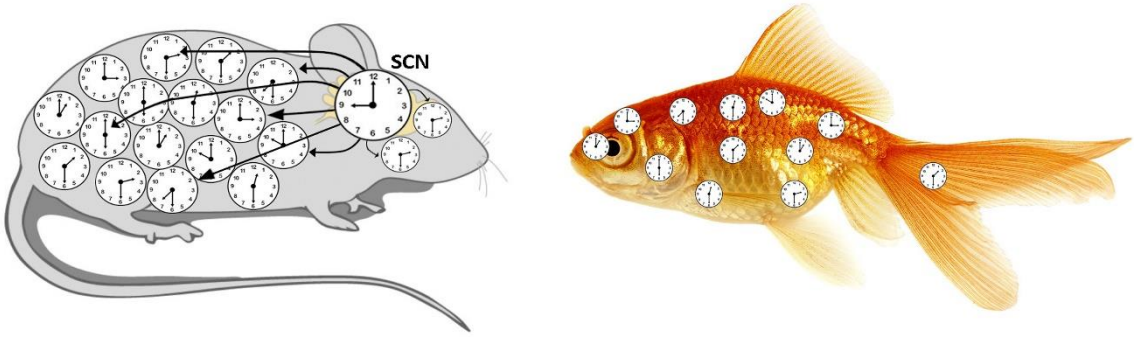


Figura 13. Organización del sistema circadiano en mamíferos (organización jerárquica con el núcleo supraquiasmático del hipotálamo – SCN – como reloj maestro) **y en peces** (red de osciladores sin jerarquización)**.**

Por su parte, en los peces teleósteos, el sistema circadiano está formado por una compleja red de osciladores distribuidos por prácticamente todos los tejidos y órganos del cuerpo de estos animales (hipotálamo, diencéfalo, glándula pineal, techo óptico, retina, hipófisis, hígado, intestino, gónadas, cabeza de riñón y piel) y en la que hasta el momento no se ha descrito la existencia de ningún reloj maestro, por lo que se considera que este sistema está menos jerarquizado que en los mamíferos (Moore y Whitmore, 2014; Sánchez-Bretaño *et al.*, 2015a; Delgado *et al.*, 2017; Isorna *et al.*, 2017; Lu *et al.*, 2018).

Esta red de relojes endógenos se sincroniza por distintos *zeitgebers* externos. Entre ellos, el más importante capaz de sincronizar los osciladores circadianos es el **ciclo luz-oscuridad** (LD, del inglés *light-dark*) y los relojes que se sincronizan por este *zeitgeber* se denominan osciladores sincronizables por la luz o LEOs (*light-entrainable oscillators*; Mendoza y Challet, 2009; Schibler *et al.*, 2015). Por ejemplo, en el caso de los peces teleósteos, la retina, la glándula pineal y algunos núcleos profundos hipotalámicos presentan fotorreceptores, que responden directamente a la luz y además se consideran LEOs (Isorna *et al.*, 2017). Sin embargo, no solamente la luz es capaz de sincronizar el sistema circadiano de los seres vivos. Así, el **ciclo alimentación-ayuno** es también un importante *zeitgeber*, especialmente para los relojes periféricos, denominándose los relojes que se sincronizan por este *zeitgeber*

osciladores sincronizables por la comida o FEOs (*feeding-entrainable oscillators*; Damiola *et al.*, 2000; Mendoza y Challet, 2009). Algunos ejemplos de órganos muy bien estudiados que responden a este tipo de *zeitgeber* son el tracto digestivo y el hígado, haciendo que sincronicen sus funciones al estado energético del animal optimizándose los procesos requeridos para la digestión, el metabolismo y la utilización y almacenaje de energía (Isorna *et al.*, 2017). Otro *zeitgeber* importante es el **ciclo de temperatura**, el cual cobra una gran importancia en los peces ya que estos animales son poiquilotermos. Por último, los **ritmos circadianos hormonales** (ya sean de melatonina o cortisol, entre otras) siempre han sido considerados como salidas rítmicas controladas y generadas por los relojes endógenos centrales y periféricos, pero últimamente está cobrando mucha importancia la idea de que estas hormonas podrían estar actuando a su vez como *zeitgebers* del propio sistema circadiano (Challet, 2015; Isorna *et al.*, 2017).

3.3 Interacciones con la homeostasis energética. Importancia de los receptores nucleares del bucle auxiliar

La **homeostasis energética** es un término que hace referencia al balance que existe entre la energía que captan los organismos a través de la ingesta (energía de entrada) y el gasto energético que se pierde para hacer frente a las necesidades del organismo (energía de salida). Una homeostasis energética adecuada tiende a generar un balance neutro, es decir, las energías de entrada y de salida se igualan, de tal manera que el organismo capta la energía necesaria para cubrir sus exigencias metabólicas. El problema viene cuando esta homeostasis energética falla hacia alguno de los dos lados produciéndose distintos tipos de enfermedades, como la obesidad, la diabetes o la esteatosis hepática, entre otras (Timper y Brüning, 2017; Godfrey y Borgland, 2019; Tutunchi *et al.*, 2019; Zhu *et al.*, 2019). En las últimas décadas, se han desarrollado multitud de trabajos en los que se evidencia que un gran número de procesos metabólicos se encuentran bajo un control circadiano en los mamíferos (Marcheva *et al.*, 2009; Asher y Schibler, 2011; Challet, 2015). Esto concuerda con el descubrimiento de la ritmicidad de multitud de genes metabólicos a nivel periférico en una gran cantidad de tejidos, tales como hígado, tejido adiposo, músculo esquelético, corazón y sistema vascular (Panda *et al.*, 2002a; Storch *et al.*, 2002; Allaman-Pillet *et al.*, 2004; Rudic *et al.*, 2005; Oishi *et al.*, 2006; Yang *et al.*, 2006; Zvonic *et al.*, 2006). Uno de los órganos más importantes es el **hígado**, en el cual existe una conexión entre el sistema circadiano y el metabolismo hepático para sincronizar el almacenaje y utilización de la energía a los ciclos diarios de luz-oscuridad (Reinke y Asher, 2016; Yang *et al.*, 2017). A pesar de toda esta ola de hallazgos, los mecanismos

intrínsecos que subyacen a la interacción entre los osciladores circadianos y el metabolismo aún están siendo investigados.

Unas de las moléculas que se postulan como principales nexos entre el metabolismo y el sistema circadiano son los **receptores nucleares** (Albrecht y Ripperger, 2018; Weikum *et al.*, 2018). Entre estos receptores nucleares que exhiben patrones circadianos de expresión se encuentran varias isoformas de REV-ERB (α y β), ROR (α, β y γ) y PPAR (α, γ y β/δ) (Yang *et al.*, 2006). Todos ellos, tal y como se indicó previamente en el apartado 3.1 de esta introducción, son genes reloj que forman parte de la maquinaria molecular complementaria al bucle principal de los osciladores endógenos, siendo dianas del heterodímero CLOCK:BMAL1 y a su vez también reguladores de este.

En los mamíferos, **REV-ERBα** interviene en una multitud de procesos metabólicos en una gran cantidad de tejidos periféricos (Marcheva *et al.*, 2009; Everett y Lazar, 2014; Vieira *et al.*, 2015). A nivel hepático, junto con REV-ERBβ, interviene en el metabolismo lipídico y glucídico, suprimiendo la gluconeogénesis y, por tanto, afectando al transporte de la glucosa hacia el torrente sanguíneo (Yin *et al.*, 2007). En el tejido adiposo participa en la diferenciación de los adipocitos durante el desarrollo, la adipogénesis, el almacenamiento de grasas y el mantenimiento de la temperatura corporal (Fontaine *et al.*, 2003; Laitinen *et al.*, 2005; Gerhart-Hines *et al.*, 2013; Vieira *et al.*, 2015). En el páncreas, REV-ERBα tiene efectos tanto en las células α productoras de glucagón como en las células β productoras de insulina, así como en la regulación de genes lipogénicos presentes en los islotes pancreáticos (Vieira *et al.*, 2012, 2013). En el músculo esquelético, este gen inhibe la diferenciación de las células musculares (en coordinación con REV-ERBβ; Welch *et al.* 2017a, b) y es un promotor del gasto energético ya que genera un aumento de la función mitocondrial en células musculares (Woldt *et al.*, 2013). Y a nivel cerebral se encarga de modular aspectos comportamentales relacionados con la sociabilidad y la ansiedad (Zhao y Gammie, 2018). Por su parte, en los peces se encuentra vagamente estudiado el papel de REV-ERB sobre la homeostasis energética, aunque debido a su gran homología con los mamíferos se ha sugerido que podría desempeñar papeles parecidos. Por ejemplo, Kopp y colaboradores (2016) determinaron que REV-ERBα regula la adipogénesis en el pez cebra, tal y como ocurre en los mamíferos.

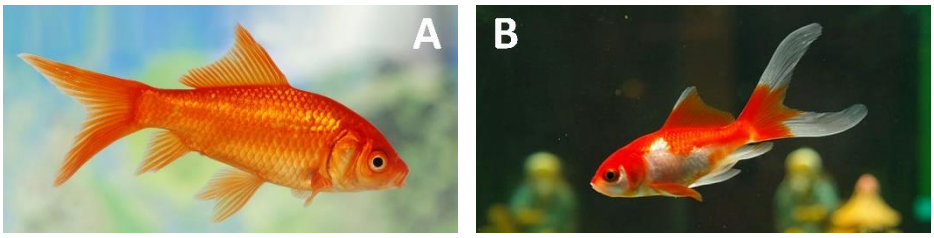
Por su parte, **RORα** se encuentra involucrado en la lipogénesis y el almacenamiento de lípidos en mamíferos (Sato *et al.*, 2004). De esta forma, la acción conjunta de REV-ERBα y RORα es fisiológicamente muy importante para mantener el control de la homeostasis del colesterol y los triglicéridos (Canaple *et al.*, 2006). Por su parte en los peces, este receptor

nuclear ha sido clonado en el pez cebra (Flores *et al.*, 2007; Katsuyama *et al.*, 2007), la carpa herbívora (*Ctenopharyngodon idellus*; Du *et al.* 2012), el bacalao del Atlántico (Lazado *et al.*, 2014), la perca china (*Siniperca chuatsi*; Wu *et al.* 2016) y el fúndulo (*Fundulus heteroclitus*; Baldwin *et al.* 2017) con una distribución tisular amplia que incluye el encéfalo y tejidos periféricos como el intestino, músculo, corazón, bazo o timo, entre otros. Sus principales funciones en los peces se han relacionado con el desarrollo embrionario de estructuras cerebelares (Katsuyama *et al.*, 2007) y el sistema inmune (Du *et al.*, 2012). Sin embargo, debido a su gran similitud en cuanto a estructura proteica y patrones de expresión con los mamíferos (Lazado *et al.*, 2014; Wang *et al.*, 2015; Wu *et al.*, 2016), el receptor nuclear RORα podría intervenir en la regulación del metabolismo glucídico y lipídico como en los mamíferos (Flores *et al.*, 2007; Baldwin *et al.*, 2017).

En cuanto a los **PPARs**, son receptores nucleares que intervienen en una gran cantidad de procesos biológicos con principal influencia sobre el metabolismo lipídico y glucídico controlando así la homeostasis energética en mamíferos (Charoensuksai y Xu, 2010; Chen y Yang, 2014). Sin embargo, cada isoforma posee distintas funciones y patrones de distribución tisular, a pesar de su alto grado de homología. Así, la principal isoforma que interviene en el sistema circadiano es **PPARα** que, como ya se indicó en el apartado 2.3, es el receptor principal al que se unen las NAEs, y destaca por la potente capacidad anorexigénica que desarrolla a través de la unión de su principal ligando, la OEA (Fu *et al.*, 2003; Sihag y Jones, 2018). Además, la regulación directa sobre la expresión de numerosos genes que intervienen en el metabolismo del colesterol y lipídico, tales como SREBP (proteína de unión del elemento de unión del esterol), FAS (sintasa de ácidos grasos) o enzimas encargadas de la β-oxidación de los ácidos grasos (Rakhshandehroo *et al.*, 2010; Bowen *et al.*, 2017). Por su parte en los peces, los PPARs son uno de los receptores nucleares más estudiados en el pez cebra (Schaaf, 2017). Focalizando nuestro estudio en la isoforma PPARα, este receptor nuclear también parece estar involucrado en el control del metabolismo lipídico a través de la modulación de diferentes genes relacionados con dicho metabolismo en distintas especies, como el pez cebra (Den Broeder *et al.*, 2015; Paredes *et al.*, 2015; Kopp *et al.*, 2016), salmón del Atlántico (Betancor *et al.*, 2014), la dorada (Paredes *et al.*, 2014), el atún común (*Thunnus thynnus*; Betancor *et al.* 2017), lenguado oliva (*Paralichthys olivaceus*; Cho HK *et al.* 2012) o el rodaballo (*Scophthalmus maximus*; Cunha *et al.* 2013).

4 EL CARPÍN: CARACTERÍSTICAS DE LA ESPECIE

El carpín o carpa dorada (*Carassius auratus*) es un pez teleósteo de agua dulce perteneciente al Orden *Cypriniformes*, Familia *Cyprinidae*, Subfamilia *Cyprininae*. Los ciprínidos comprenden más de 2000 especies agrupadas en más de 200 géneros que se encuentran ampliamente distribuidas en ríos de África, Europa, Asia y Norteamérica (desde el norte de Canadá hasta el sur de México). El carpín es una especie originaria de China que comenzó a domesticarse hace más de mil años, se introdujo en Japón en el siglo XVI, desde donde fue exportado a Europa en el siglo XVII (Kottelat y Freyhof, 2007). Debido a la gran cantidad de cruces selectivos que han tenido lugar a lo largo de su cría en cautividad se han desarrollado más de 300 variedades de la especie, con diferentes formas, hábitats y modos de vida. Actualmente, la mayoría de las variedades se cultivan principalmente como peces ornamentales, aunque también son muy utilizados en investigación (Kottelat y Freyhof, 2007). En la presente Tesis Doctoral se han utilizado ejemplares de las variedades común y cometa (**Fotografía 1**).



Fotografía 1. Ejemplares adultos de carpín (*Carassius auratus*) variedad común (A) y cometa (B).

Morfológicamente, este ciprínido presenta un cuerpo compacto con una coloración originalmente pardo-amarillenta o anaranjada con reflejos blanco-plateados por los costados y el vientre (**Figura 14**). Los adultos suelen medir entre 10 y 20 cm de longitud, aunque es posible encontrar ejemplares que llegan a medir 45 cm. Presenta cinco juegos de aletas con un

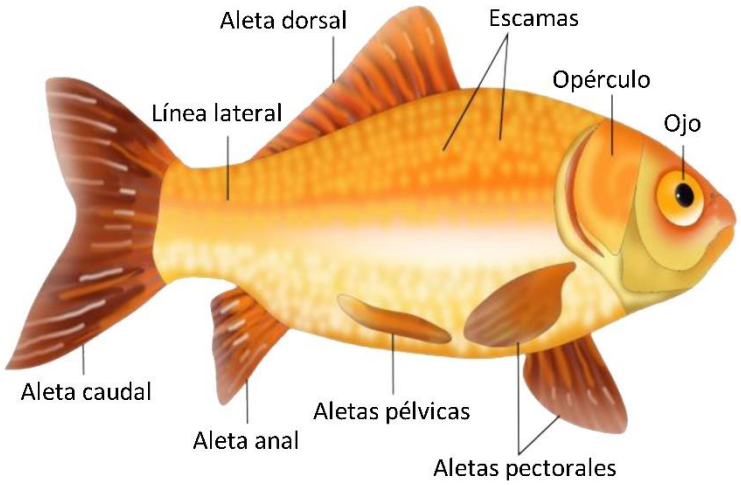


Figura 14. Esquema de la morfología externa del carpín. Modificada de Blanco *et al.* (2018).

patrón de disposición típico de la mayoría de los miembros de la Familia *Cyprinidae*: una aleta dorsal de base amplia con presencia de espinas óseas, una gran aleta caudal homocerca al final de un pedúnculo ancho y corto, una aleta anal situada posteriormente al ano del animal, un par de aletas pélvicas cortas y anchas a cada lado del animal y un par de aletas pectorales ubicadas también a cada lado justo detrás del opérculo

La anatomía interna de este pez teleósteo se muestra en la **Figura 15**. El tracto digestivo de estos animales comienza en la boca y la faringe, la cual se encuentra conectada con la musculatura de la cabeza y contiene los dientes faríngeos (cuatro filas a cada lado) unidos a los huesos faríngeos posteriores, gracias a los cuales tritura el alimento. Como la mayoría de los peces, el carpín no secreta enzimas digestivas en esta región del tracto gastrointestinal. La faringe se continúa con un esófago corto y este conecta con el intestino a través del esfínter intestinal. La peculiaridad más llamativa de esta especie es la carencia de estómago, de tal forma que tras esta constricción circular aparece un ensanchamiento del intestino que se denomina bulbo intestinal. Esta región se distingue del resto del tubo digestivo por su mayor anchura y por el mayor grosor de sus capas musculares, lo que le otorga una gran capacidad de expansión (McVay y Kaan, 1940; Kapoor *et al.*, 1976). En el final de este bulbo intestinal es donde desemboca la vesícula biliar, vertiéndose las secreciones biliares para iniciar la digestión enzimática. Después se extiende un intestino de gran longitud que puede diferenciarse en tres partes: intestino anterior, intestino medio e intestino posterior, el cual se comunica con el exterior a través del ano. Asociado a todo este tracto gastrointestinal, cabe destacar la presencia de un hígado con anatomía difusa, formado por estrechos lóbulos hepáticos que se extienden alrededor de toda la longitud del intestino, y en el que se encuentra embebido un rudimentario páncreas en forma de células dispersas (Sarbahi, 1951). Durante la presente Tesis

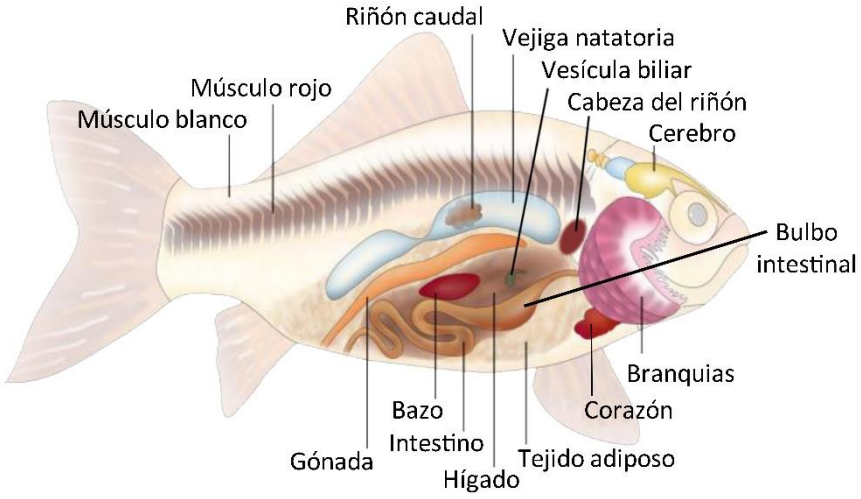


Figura 15. Esquema de la anatomía interna y principales órganos del carpín. Modificada de Blanco *et al.* (2018).

Doctoral, nos referiremos a esta organización estructural que engloba al hígado y al páncreas con el término de hígado.

El carpín prefiere hábitats eutróficos en aguas poco profundas de lagunas y ríos con corriente lenta que presenten fondos blandos y ricos en vegetación. Se aclimata a aguas con una temperatura templada o fría (10-23°C) y pH próximos a la neutralidad (7.0-7.5) (Kottelat y Freyhof, 2007), aunque es capaz de adaptarse a condiciones más extremas como temperaturas elevadas o hipoxia (Muus y Dahlström, 1981). Es un animal omnívoro y bentónico que se caracteriza por una natación constante buscando y persiguiendo la comida. En la naturaleza se alimenta fundamentalmente de organismos planctónicos, pequeños crustáceos, moluscos, gusanos, larvas de insectos y plantas acuáticas (Muus y Dahlström, 1981). Su crecimiento por lo general es rápido, aunque se encuentra principalmente determinado por la cantidad de alimento que pueda obtener de su entorno. En términos generales, el carpín es un teleósteo diurno cuyos máximos de actividad locomotora se desarrollan durante la fotofase del ciclo diario (Aranda *et al.*, 2001). Teniendo en cuenta la importancia que tiene la alimentación como factor sincronizador, los carpines presentan una marcada actividad anticipatoria al alimento (FAA, del inglés *food anticipatory activity*) caracterizada por un aumento del movimiento entre 2 y 3 horas previas al momento de la alimentación (Isorna *et al.*, 2017).

Este ciprínido alcanza la madurez sexual a partir de los 8-10 meses de edad. La reproducción o freza se produce en aguas con una densa vegetación entre los meses de mayo y julio, pudiendo verse modificado en función de la temperatura del agua. Dependiendo del tamaño de la hembra, esta puede llegar a poner entre 200.000 y 400.000 huevos que deposita adheridos a las plantas acuáticas de las orillas. Estos huevos tardarán en eclosionar entre 3 y 8 días, también dependiendo principalmente de la temperatura del agua (Muus y Dahlström, 1981).

El carpín es una especie de pez teleósteo ampliamente utilizada en el ámbito de la investigación debido a la facilidad de su manejo, su pequeño tamaño y su sencillo mantenimiento, así como la capacidad de adaptación a una gran variedad de condiciones experimentales en el laboratorio (Kottelat y Freyhof, 2007; Blanco *et al.*, 2018). Así, en la última década, nuestro grupo de investigación "Neuroendocrinología de Peces" se ha especializado en el estudio de la fisiología de este pez teleósteo, focalizándose fundamentalmente en el estudio sobre la regulación de la ingesta, el estrés y el sistema circadiano.


OBJETIVOS

OBJETIVOS

Según la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO, 2018), la acuicultura se presenta como uno de los sectores de producción de alimentos con mayor capacidad de crecimiento que permitirán mantener la proporción de pescado en la dieta mundial, estimando que para el año 2030 más del 62% de los alimentos acuáticos procederán de la acuicultura. Además, la acuicultura es un pilar esencial del Crecimiento Azul que propugnan la Unión Europea y España para alcanzar sus objetivos por una piscicultura de calidad y sostenible (Apromar, 2018). Teniendo en cuenta que en el cultivo de peces el gasto en alimentación representa uno de los mayores costes económicos, uno de los principales retos hoy en día en la industria de la acuicultura para conseguir la sostenibilidad de dicha actividad es el diseño de estrategias de alimentación más eficaces, así como el control del bienestar de estos animales. Para poder hacer frente a ambos desafíos, es imprescindible tener un conocimiento lo más exhaustivo posible de la regulación de la homeostasis energética y la influencia de los ritmos de alimentación sobre ella en los peces.

Tal y como se comentó en la Introducción, el control de la ingesta es un proceso complejo y multifactorial, resultante de la integración a nivel central de información neuroendocrina, metabólica y circadiana (Delgado *et al.* 2017). Actualmente se conocen un gran número de señales neuroendocrinas implicadas en la regulación de la homeostasis energética en los peces, como neuropéptidos, hormonas y monoaminas. Sin embargo, se ha prestado poca atención a moléculas de otra naturaleza, como los derivados lipídicos N-aciletanolaminas, objeto de estudio de la presente Tesis Doctoral. El interés en estas moléculas reside además en el hecho de que una de sus principales vías de acción es a través del receptor nuclear PPARα, que conecta el metabolismo con la maquinaria molecular controlando los osciladores

endógenos en los mamíferos, aunque apenas se sabe nada de si existe esta conexión con el sistema circadiano en los peces.

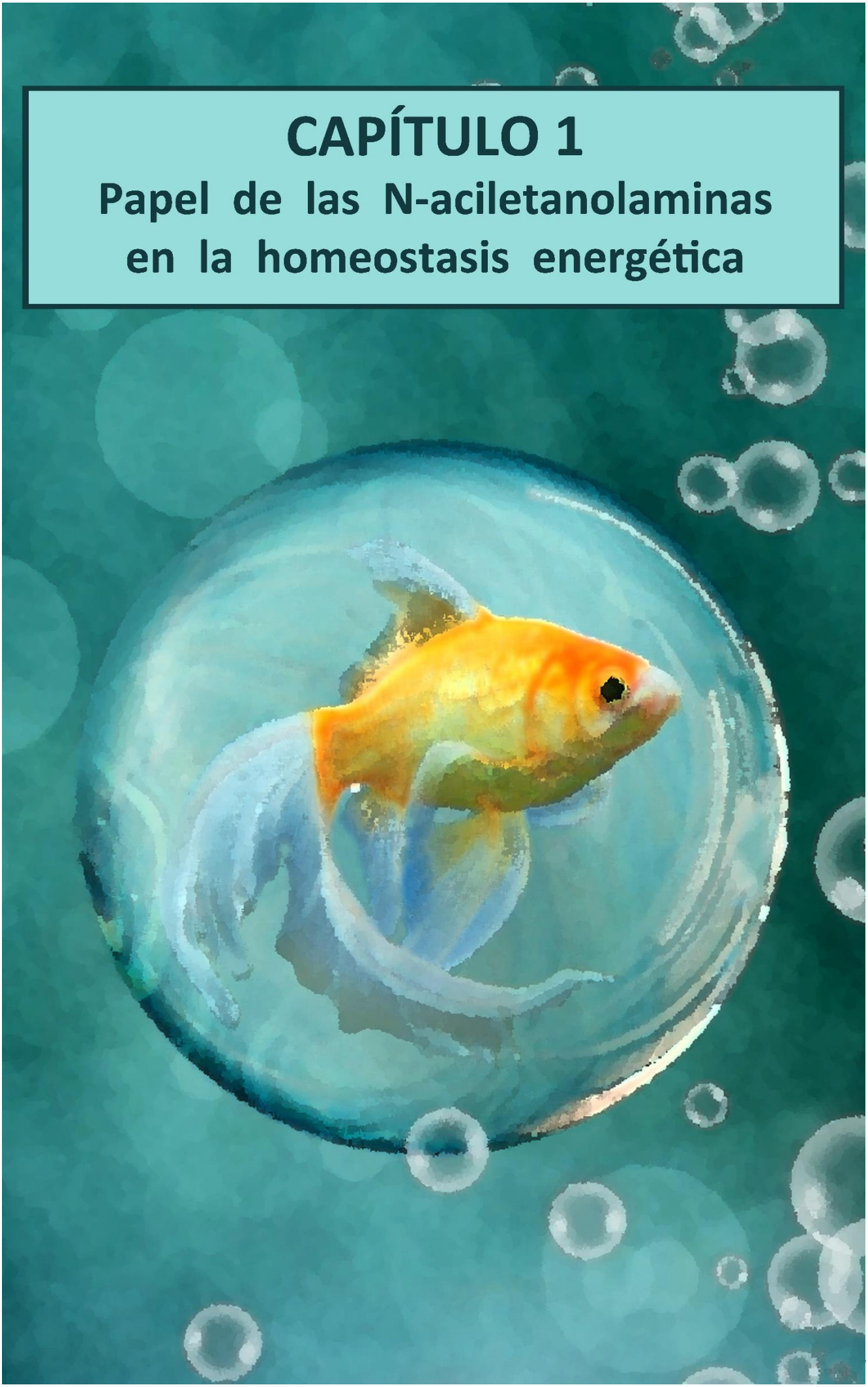
Por todo ello, el objetivo general de la presente Tesis Doctoral es el estudio del papel de las NAEs en la homeostasis energética de los peces, así como ahondar en la relación entre el sistema circadiano (y la homeostasis temporal que este controla) y dicha homeostasis energética, utilizando como modelo el carpín. Para abordar este objetivo general, se han desarrollado los siguientes objetivos específicos:

1. Caracterizar componentes esenciales del sistema de las NAEs en los peces, mediante el estudio de los perfiles diarios de OEA, PEA y SEA, sus precursores, enzimas de síntesis y degradación, y del principal receptor PPARα, en diferentes tejidos gastrointestinales y cerebrales.
2. Determinar el papel de las NAEs en la regulación de la ingesta a corto plazo en los peces, evaluando si se produce una regulación en el contenido de OEA, PEA y SEA por el estado nutricional del animal. Además, investigaremos el efecto de la administración aguda de PEA y SEA en la ingesta y las posibles interacciones con otros reguladores de la ingesta.
3. Estudiar los posibles efectos de la OEA y la PEA sobre otros componentes de la homeostasis energética, como el peso corporal, la actividad locomotora y el metabolismo hepático de lípidos y glúcidos, así como identificar posibles interacciones de estas NAEs con el sistema circadiano.
4. Averiguar si el horario de alimentación y/o el ciclo de luz-oscuridad determinan los ritmos de expresión de genes reloj en el hipotálamo y el hígado del carpín, dos tejidos clave en la regulación de la ingesta y el metabolismo en peces, así como identificar posibles salidas rítmicas relacionadas con la homeostasis energética de dichos osciladores.
5. Investigar si los ritmos de expresión de algunos receptores nucleares que forman parte del bucle auxiliar de los osciladores endógenos y que tienen un papel relevante en el control de la homeostasis energética, son salidas directas del bucle principal de reloj o, por el contrario, dependen más de la luz o la ingesta que puedan actuar enmascarando los ritmos.
6. Conocer el efecto de una disrupción temporal (desacoplamiento entre los principales sincronizadores ciclo luz-oscuridad y ciclo alimentación-ayuno) sobre los ritmos de expresión de genes reloj en el hipotálamo, el hígado y la glándula interrenal en peces, así como sobre los ritmos de la principal hormona indicadora de estrés, el cortisol.


RESULTADOS

CAPÍTULO 1

Papel de las N-aciletanolaminas en la homeostasis energética



CAPÍTULO 1

Papel de las N-aciletanolaminas en la homeostasis energética

1.1 Efectos del factor de saciedad oleiletanolamida en el metabolismo hepático de los lípidos y de la glucosa en el carpín.

The satiety factor oleoylethanolamide impacts hepatic lipid y glucose metabolism in goldfish.

Journal of Comparative Physiology B **(2016) 186:1009–1021.**

doi: 10.1007/s00360-016-1009-x

1.2 Primeras evidencias acerca del papel de la palmitoiletanolamida sobre la homeostasis energética en peces.

First evidence on the role of palmitoylethanolamide in energy homeostasis in fish.

Hormones & Behavior **(2019).**

En revisión

1.3 Perfiles diarios de las N-aciletanolaminas en el cerebro y tracto gastrointestinal del carpín: posible papel de la alimentación.

Diurnal profiles of N-Acylethanolamines in goldfish brain y gastrointestinal tract: Possible role of feeding.

Frontiers in Neuroscience **(2019) 13:450.**

doi: 10.3389/fnins.2019.00450

Los principales resultados obtenidos en los tres trabajos que configuran el Capítulo 1 de la presente Tesis Doctoral se recogen en la **Figura 16**.

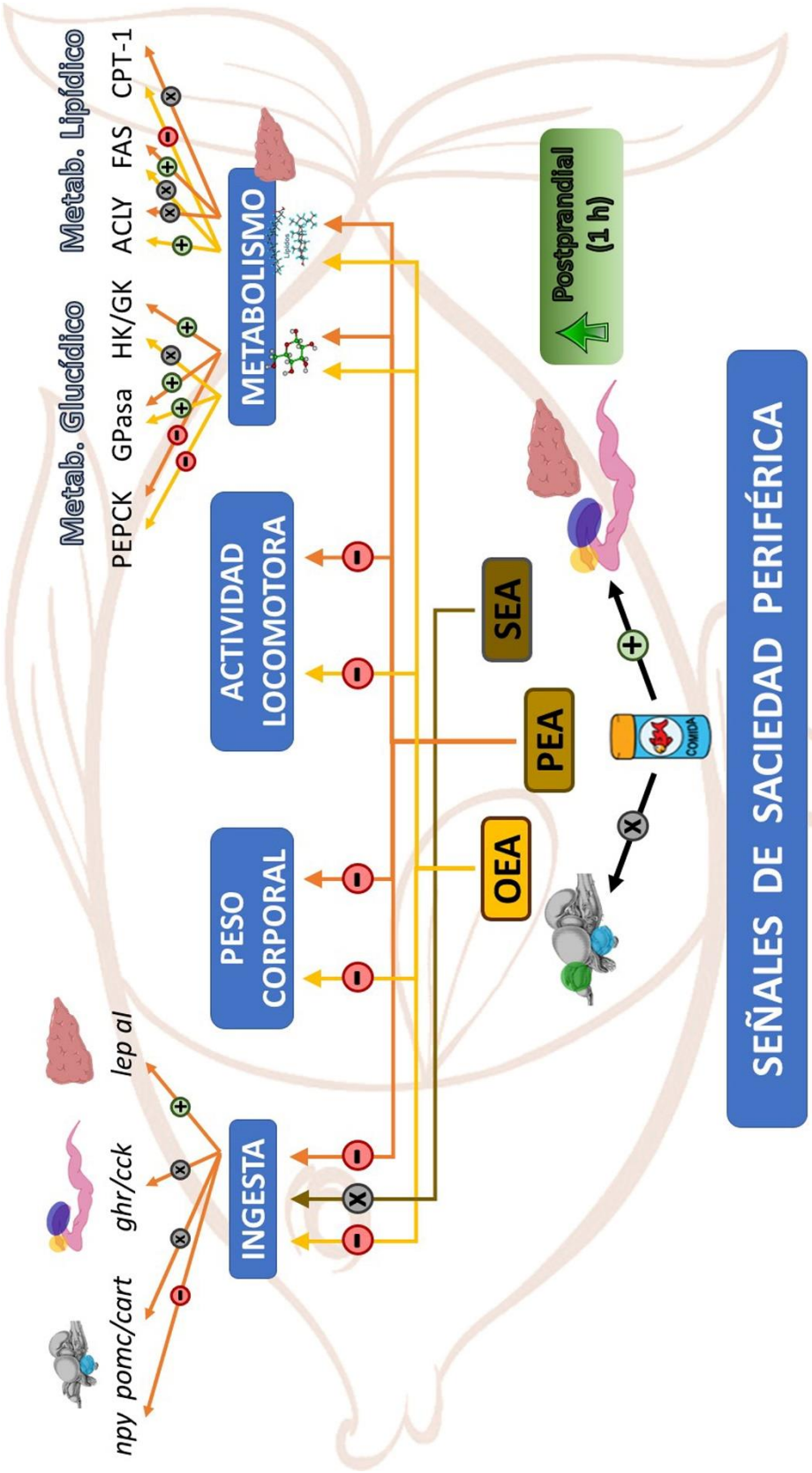


Figura 16. Resumen de los resultados del Capítulo 1. ACLY, ATP-citrato liasa; *cart*, péptido regulado por cocaína y anfetamina; *cck*, colecistocinina; CPT-1, carnitina palmitoiltransferasa 1; FAS, ácido graso sintasa; *ghr*, ghrelina; GK, glucoquinasa; GPasa, glucógeno fosforilasa; HK, hexoquinasa; *lep al*, leptina al; *npy*, neuropéptido Y; PEPCK, fosfoenolpiruvato carboxiquinasa; *pomc*, proopiomelanocortina; OEA, oleiletanolamida; PEA, palmitoiletanolamida; SEA, estearoiletanolamida. ⊕, estimulación; ⊖, inhibición; ⊗, no efecto.

J Comp Physiol B (2016) 186:1009–1021
DOI 10.1007/s00360-016-1009-x

ORIGINAL PAPER

The satiety factor oleoylethanolamide impacts hepatic lipid and glucose metabolism in goldfish

Miguel Gómez-Boronat[1] · Cristina Velasco[2] · Esther Isorna[1] · Nuria De Pedro[1] · María J. Delgado[1] · José L. Soengas[2]

Received: 8 March 2016 / Revised: 25 May 2016 / Accepted: 3 June 2016 / Published online: 8 June 2016

Abstract Oleoylethanolamide (OEA) is an acylethanolamide synthesized mainly in the gastrointestinal tract with known effects in mammals on food intake and body mass through activation of peroxisome proliferator-activated receptor type α (PPARα). Since we previously demonstrated that acute treatment with OEA in goldfish resulted in decreased food intake and locomotor activity, as in mammals, we hypothesize that OEA would be involved in the control of energy metabolism in fish. Therefore, we assessed the effects of acute (for 6 h) and chronic (for 11 days) treatments with OEA (5 μg g^{-1} body mass) on metabolite concentrations and enzyme activities related to glucose and lipid metabolism in liver of goldfish (*Carassius auratus*). In the chronic treatment, OEA impairs the increase in body mass and reduces locomotor activity, without any signs of stress. The lipolytic capacity in liver decreased after both acute and chronic OEA treatments, whereas lipogenic capacity increased after acute and decreased after chronic treatment with OEA. These results are different from those observed to date in mammalian adipose tissue, but not so different from those known in liver, and might be attributed to the absence of changes in the expression of *pparα*, and/or to the increase in the expression of the clock gene *bmal1a* after chronic OEA treatment. As for glucose metabolism, a clear decrease in the capacity of hepatic tissue to use glucose was observed in OEA-treated fish. These results support an important role for OEA in the regulation of liver lipid and glucose metabolism, and could relate to the metabolic changes associated with circadian activity and the regulation of food intake in fish.

Keywords Oleoylethanolamide (OEA) · Goldfish · Liver · Lipid metabolism · Glucose metabolism · Body mass

Communicated by I. D. Hume.

✉ José L. Soengas
jsoengas@uvigo.es

[1] Departamento de Fisiología (Fisiología Animal II), Facultad de Biología, Universidad Complutense de Madrid, 28040 Madrid, Spain

[2] Laboratorio de Fisioloxía Animal, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía, Edificio de Ciencias Experimentais, Universidade de Vigo, 36310 Vigo, Spain

Introduction

In mammals, viscerosensory signals from the gut contribute to build a complex network of neural and hormonal signals that converge in the brain to control feeding behaviour and energy balance. One family of phospholipid-derived signalling molecules playing an important role in this network is the acylethanolamides including oleoylethanolamide (OEA) (Piomelli 2013; Romano et al. 2015). OEA is a structural analogue of the endocannabinoid arachidonoylethanolamide (anandamide or AEA), although in contrast to AEA, OEA does not bind to or activate the endocannabinoid 1 (CB1) receptor (Serrano et al. 2008). Instead, OEA induces reduction of food intake (Fu et al. 2005; Serrano et al. 2008, 2011; Thabuis et al. 2011; Romano et al. 2013; Suárez et al. 2014; Azari et al. 2014) and body mass (Guzmán et al. 2004; Fu et al. 2005; Serrano et al. 2008; Thabuis et al. 2011; Suárez et al. 2014) through activation of peroxisome proliferator-activated receptor type α (PPARα). This anorectic effect is not due to stress, and the response is also accompanied by a decreased locomotor activity in mammals (Rodríguez de Fonseca et al. 2001; Proulx et al. 2005).

OEA is synthesized in a variety of cells, including neurons, astrocytes, enterocytes, and adipocytes, and its production is regulated by nutritional status. In the small intestine, OEA levels are decreased by food deprivation and increased upon refeeding in rodents (rats and mice; Rodríguez de Fonseca et al. 2001; Piomelli 2013) and reptiles (*Python molurus*; Astarita et al. 2006). Recently, a feeding-induced OEA mobilization in intestine has been also described in fish, in particular, in goldfish (*Carassius auratus*, Linnaeus; Tinoco et al. 2014). Moreover, a possible role of OEA as an intestinal fat sensor has been suggested since OEA levels in this tissue can be modified by the fat dietary content (Piomelli 2013; Liu et al. 2014). These results suggest that endogenous OEA in vertebrates can act as a satiety signal, which is generated in intestine in response to fat ingestion, and signals to the central nervous system (Romano et al. 2015).

Besides the control of food intake, OEA seems to be involved in the peripheral control of energy balance. Thus, in rodent adipocytes and adipose tissue, acute or chronic OEA treatments reduced lipogenesis, and enhanced lipolysis and fatty acid oxidation (Guzmán et al. 2004; Fu et al. 2005; Serrano et al. 2008; Thabuis et al. 2011; Suárez et al. 2014). In other tissues, such as the liver, there are fewer studies with contradictory results regarding effects of OEA on lipogenesis, with an absence of effects in two studies (Fu et al. 2005; Serrano et al. 2008), or a decrease or increase observed in another (Thabuis et al. 2011). In these metabolic studies, the parameters assessed were metabolic rates (Guzmán et al. 2004; Thabuis et al. 2011), and in some cases, mRNA abundance of several enzymes involved in lipid metabolism (Guzmán et al. 2004; Fu et al. 2005; Serrano et al. 2008; Thabuis et al. 2011). However, as far as we are aware, in none of these studies were changes in enzymatic activities assessed yet. As for glucose metabolism, OEA treatment decreased glucose tolerance in rat (González-Yanes et al. 2005), but little information is available regarding effects of OEA in tissues such as the liver (Thabuis et al. 2011).

In fish, we previously demonstrated for the first time that intraperitoneal (ip) acute treatment with OEA in goldfish resulted in decreased food intake and locomotor activity (Tinoco et al. 2014). The mechanisms involved in these OEA actions have been partially elucidated in fish, being mediated, at least in part, through interactions with ghrelin and the serotoninergic system (Tinoco et al. 2014). In the same study, we observed decreased levels of triglyceride in plasma. Apart from these data, there is no evidence in fish on the possible effects of OEA in energy metabolism. We hypothesize that OEA is involved in the control of energy metabolism in fish. Thus, we aimed to assess the effects of acute and chronic treatment with OEA on glucose and lipid metabolism in the liver of goldfish, in which we previously described OEA effects on the regulation of food intake (Tinoco et al. 2014), a process also known in fish to be regulated by lipid and glucose metabolism (Soengas 2014).

Therefore, we intraperitoneally (ip) treated goldfish with OEA (5 μg g^{-1} body mass) acutely or chronically and assessed (1) metabolite levels in plasma (fatty acid, triglyceride, glucose) and liver (fatty acid, triglyceride, total lipid, glucose, glycogen, and lactate), (2) the activity of enzymes involved in lipid metabolism in liver, such as ATP-citrate lyase (ACLY), fatty acid synthase (FAS), 3-hydroxyacyl-CoA dehydrogenase (HOAD), and carnitine palmitoyltransferase-1 (CPT-1), (3) the activity of enzymes involved in glucose metabolism in liver, such as hexokinase (HK), glucokinase (GK), pyruvate kinase (PK), 6-phosphofructo 1-kinase (PFK), fructose 1,6-bisphosphatase (FBPase), phospho(enol)pyruvate carboxykinase (PEPCK), glycogen phosphorylase (GPase), glycogen synthase (GSase), glucose 6-phosphatase (G6Pase), and glucose-6-phosphate dehydrogenase (G6PDH), and (4) expression of genes involved in putative OEA regulatory action on energy metabolism, such as *pparα* (Guzmán et al. 2004) and *bmal1a* (Shimba et al. 2005). Moreover, modifications in body mass and locomotor activity throughout the time-course of chronic administration of OEA were also evaluated.

Materials and methods

Animals

Goldfish were obtained from a local commercial supplier in Madrid (Spain). Fish were kept in 60 litre aquaria with filtered and aerated fresh water (21 ± 2 °C) in a temperature-controlled room under a 12 h light:12 h dark photoperiod (lights on at 8 am). Animals were fed once daily at 10:00 with 1 % body mass commercial dry pellets (32.1 % crude protein, 5 % crude fat, 1.9 % crude fibre, 6.8 % crude ash, 5.1 % water and the rest nitrogen free extract; sera pond Biogram, Heinsberg, Germany). Animals were maintained under these conditions for at least 2 weeks prior to experimental use. The aquaria walls were covered with an opaque paper to minimize external interference during the experiments. The experiments described comply with the Guidelines of the European Union Council (2010/63/UE) and the Spanish Government (RD53/2013) for the use of animals in research, and were approved by the Animal Experimentation Committee of Complutense University (O.H.-UCM-25-2014) and the Community of Madrid (PROEX 107/14).

OEA administration

OEA (Sigma Chemical, Madrid, Spain) was dissolved in a vehicle formed by 5 % Tween 20, 5 % polyethyleneglycol (Sigma Chemical) and 90 % teleost saline (20 mg Na_2CO_3/100 ml of 0.6 % NaCl). Fish were anaesthetized in water containing buffered tricaine methanesulfonate (MS-222, 0.14 g l^{-1}; Sigma Chemical) before being handled, injected or sampled, to minimize stress. The ip injections were performed using 1 ml syringes and 0.3 mm Microlance needles (Lab-Center, Madrid, Spain), close to the ventral midline posterior to the pelvic fins (Tinoco et al. 2014). Fish were ip injected with 10 μl g^{-1} of vehicle alone (control) or containing OEA (5 μg g^{-1} body mass, OEA group). The OEA dose was chosen based on previous experiments in goldfish (Tinoco et al. 2014). After the ip injections, fish were transferred to the experimental aquaria with anaesthetic-free water, where swimming activity and equilibrium recovered within 1–2 min.

Experimental design

Acute treatment

Fish (8–13 g body mass) were divided into two groups ($N = 10$ fish/group): control and OEA (5 μg g^{-1} body mass). The day of the experiment, fish were not fed and received one ip injection with vehicle alone (control, CON) or containing OEA at the scheduled feeding time (10:00). After 6 h all fish were sampled. Thus, fish were anaesthetized (MS-222, 0.14 g l^{-1}) and blood samples were collected by heparinized syringes from the caudal vein. Plasma samples were obtained after blood centrifugation, frozen on liquid nitrogen, and stored at −80 °C until further assay. Then, animals were killed by anaesthetic overdose (MS-222, 0.28 g l^{-1}) followed by spinal cord section. Liver was dissected on ice, frozen in liquid nitrogen and immediately stored at −80 °C until analysis.

Chronic treatment

Two groups ($N = 10$ fish/group) of goldfish (13–20 g body mass) were daily ip injected during 11 days with vehicle alone (control, CON) or containing OEA (5 μg g^{-1} body mass). This experimental period was based on previous studies employing other feeding regulators, in which significant modifications in body mass and locomotor activity were observed in this species (De Pedro et al. 2006; Azpeleta et al. 2010). Fish were fed once daily at scheduled feeding time (10:00) and injected 3 h after feeding time (13:00). Body mass and locomotor activities were recorded daily throughout the experiment. On the last day of the experimental period, fish (under 48 h fasting) were sampled as described above.

Analytical techniques

Locomotor activity determination

Locomotor activity was recorded in groups of five or six fish using infrared photocells (OMRON E3S-AD12, Osaka, Japan) fixed on the aquaria wall, as previously described in the same species (Azpeleta et al. 2010). The photocells continuously emitted an infrared light beam, which was interrupted each time fish swam in that zone, generating an output signal. The number of light beam interruptions was automatically counted and stored every 10 min by specific software (Micronec, Spain). Locomotor activity indicates the mean ± s.e.m. of the activity recorded through 24 h periods over the 11 days of treatment.

Assessment of plasma and tissue metabolites

Plasma concentration of fatty acid, triglyceride and glucose was determined enzymatically using commercial kits (Wako Chemicals, Neuss, Germany, for fatty acid; Spinreact, Barcelona, Spain, for triglyceride and glucose) adapted to a microplate format. Plasma cortisol concentration was assessed by ELISA using a commercial kit (Demeditec, Kiel-Wellsee, Germany).

Samples used to assess liver metabolite concentration were homogenized by ultrasonic disruption in 7.5 vols of ice-cooled 0.6 N perchloric acid and neutralized with 7.5 vols of ice-cooled 1 N potassium bicarbonate. The homogenate was centrifuged (4 min at 13,500*g*, 4 °C) and the supernatant assayed for tissue metabolites. Hepatic concentration of fatty acid, total lipid, triglyceride, glucose and lactate was determined enzymatically using commercial kits (Wako for fatty acid; Spinreact for total lipid, triglyceride, and lactate; Biomérieux, Grenoble, France, for glucose) adapted to a microplate format. Liver glycogen concentration was assessed using the method of Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) and tissue glucose concentration was determined with a commercial kit (Biomérieux).

Assessment of enzyme activities

Samples for enzyme activities were homogenized by ultrasonic disruption in 9 vols of ice-cooled-buffer consisting of 50 mM imidazole (pH 7.0), 5 mM EDTA, 5 mM EGTA, 15 mM β-mercaptoethanol, 100 mM potassium fluoride, and a protease inhibitor cocktail (Sigma Aldrich). The homogenate was centrifuged (10 min at 900*g*, 4 °C) and the supernatant immediately used for enzyme assays. Enzyme activities were determined using a microplate reader Infinite 200 Pro (Tecan, Männedorf, Switzerland)

and 96-well microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm, and, in the case of CPT-1 activity, the increase of 5,5′-dithiobis(2-nitrobenzoic acid)-CoA (DTNB) complex at 412 nm. The reactions were started by the addition of supernatant (10–50 µl) at a pre-established protein concentration, omitting the substrate in the control wells (final volume 180–295 µl), and allowing the reactions to proceed at 37 °C for pre-established times (3–25 min). Enzyme activities are expressed per mg protein, which was assayed according to the bicinchoninic acid method with bovine serum albumin (Sigma Aldrich) as standard. Enzyme activities were assessed at maximum rates by preliminary tests to determine the optimal substrate concentrations by adapting to goldfish methods previously developed for rainbow trout (Polakof et al. 2007a, b, 2008a, b, c; Librán-Pérez et al. 2012, 2013a, b). ACLY (EC 4.1.3.8) activity was assessed in a Tris–HCl buffer (50 mM, pH 7.8) containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM sodium citrate, 10 mM β-mercaptoethanol, 5 mM ATP, 0.3 mM NADH, 7 U ml^{-1} malate dehydrogenase, and 300 µM Coenzyme A (omitted for controls). FAS (EC 2.3.1.85) activity was assessed in a phosphate buffer (0.1 mM K_2HPO_4 and 0.1 mM KH_2PO_4, pH 6.5) containing 0.1 mM NADPH, 25 µM acetyl-CoA, and 40 µM malonyl-CoA (omitted for controls). HOAD (EC 1.1.1.35) activity was assessed in an imidazole buffer (50 mM, pH 7.6) containing 0.3 mM NADH, and 110 µM acetoacetyl-CoA (omitted for controls). CPT-1 activity (EC 2.3.1.21) was assessed in a Tris–HCl buffer (75 mM, pH 8.0) containing 1.5 mM EDTA, 0.25 mM DTNB, 35 µM palmitoyl-CoA, and 1 mM L-carnitine (omitted for controls). HK (EC 2.7.1.1) and GK (EC 2.7.1.2) activities were assessed in a Tris–HCl buffer (80 mM, pH 8.0) containing 10.2 mM KCl, 37.5 mM $MgCl_2$, 11.5 mM KH_2PO_4, 20 mM $NaHCO_3$, 4 mM EDTA, 2.6 mM DTT, 2 mM $NADP^+$, 7 mM ATP, 0.13 U ml^{-1} glucose 6-phosphate dehydrogenase, 0.13 U ml^{-1} 6-phosphogluconate dehydrogenase, and 1 mM (HK) or 20 mM (GK) D-glucose (omitted for controls). PK activity (EC 2.7.1.40) was assessed in an imidazole buffer (50 mM, pH 7.4) containing 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM ADP, 0.15 mM NADH, 21 U ml^{-1} lactate dehydrogenase, and 2 mM phospho(enol)pyruvate (omitted for controls). PFK activity (EC 2.7.1.11) was assessed in an imidazole buffer (100 mM, pH 8.25) containing 50 mM KCl, 5 mM $MgCl_2$, 4 mM $(NH_4)_2SO_4$, 1 mM ATP, 0.15 mM NADH, 0.5 U ml^{-1} aldolase, 5 U ml^{-1} triosephosphate-isomerase, 1 U ml^{-1} α-glycerophosphate dehydrogenase, and 20 mM D-fructose 6-phosphate (omitted for controls). FBPase activity (EC 3.1.3.11) was assessed in an imidazole buffer (85 mM, pH 7.7) containing 5 mM $MgCl_2$, 0.5 mM $NADP^+$, 2.5 U ml^{-1} 6-phosphoglucose isomerase, 0.8 U ml^{-1} glucose 6-phosphate dehydrogenase, and 7 mM fructose 1,6-bisphosphate (omitted for controls). PEPCK activity (EC 4.1.1.32) was assessed in a Tris–HCl buffer (50 mM, pH 7.5) containing 1 mM $MnCl_2$, 20 mM $NaHCO_3$, 1.5 mM phospho(enol)pyruvate, 0.3 mM NADH, 1.7 U ml^{-1} malate dehydrogenase, and 4 µM 2′-deoxyguanosine-5-diphosphate (omitted for controls). GPase activity (EC 2.4.1.1) was assessed in a phosphate buffer (50 mM, pH 7.0) containing 27 mM $MgSO_4$, 24.2 mM EDTA, 2.5 mM AMP, 0.5 mM $NADP^+$, 1.7 U ml^{-1} phosphoglucomutase, 6.8 U ml^{-1} glucose 6-phosphate dehydrogenase, 10 µM α-D-glucose 1,6-bisphosphate, and 60 mg ml^{-1} glycogen (omitted for controls). GSase activity (EC 2.4.1.11) was assessed in an imidazole buffer (50 mM, pH 7.5) containing 150 mM KCl, 15 mM $MgCl_2$, 2 mg ml^{-1} glycogen, 1.5 mM phospho(enol)pyruvate, 0.3 mM NADH, 4.6 mM D-glucose 6-phosphate, 1.4 U ml^{-1} lactate dehydrogenase, 1.4 U ml^{-1} pyruvate kinase, and 5 mM uridine diphosphoglucose (omitted for controls). G6Pase activity (EC 3.1.3.9) was assessed in an imidazole buffer (100 mM, pH 6.5) containing 50 mM D-glucose 6-phosphate (omitted for controls). G6PDH activity (EC 1.1.1.49) was assessed in an imidazole buffer (78 mM, pH 7.7) containing 5 mM $MgCl_2$, 0.5 mM $NADP^+$, and 2 mM D-glucose 6-phosphate (omitted for controls).

Gene expression analysis

Bmal1a and *pparα* gene expression was measured by real-time quantitative PCR (RT-qPCR), using elongation factor-1 alpha (*ef-1α*) as a reference gene as previously described (Sánchez-Bretaño et al. 2016). Specific primers and Gene Data Bank Reference Numbers are shown in Table 1. RNA extraction (TRI® Reagent method, Sigma Chemical), DNase treatment (Promega, Madison, USA), cDNA synthesis (Superscript Reverse II Transcriptase, Invitrogen, Carlsbad, USA) and real-time PCR reactions (iTaqTM SYBR® Green Supermix in a CFX96TM Real-Time System, Biorad Laboratories, Hercules, USA) were carried out following manufacturer instructions with minor modifications (Sánchez-Bretaño et al. 2016). 0.5 µg of DNase treated RNA were retrotranscribed. PCR reactions were developed by duplicated in a final volume of 10 µl (1 µl of cDNA per sample). PCR conditions were 30 s at 95 °C, and 40 cycles consisting of 5 s at 95 °C and 30 s at 60 °C for all genes. Calibration curves were made with serial dilutions of cDNA (by triplicate), exhibiting efficiencies around 100 %. Specificity of amplifications was checked by melting curves and also by the size of PCR products in an agarose gel. Negative controls included replacement of cDNA by water and use of non-retrotranscribed RNA. The relative mRNA expression

Table 1 Primers sequences used in the RT-qPCR assays

Target gene	Accession number	Primer sequences 5′–3′		Product (bp)
ef-1α	AB056104.1	Forward	CCCTGGCCACAGAGATTTCA	101
		Reverse	CAGCCTCGAACTCACCAACA	
bmal1a	KF840401.1	Forward	AGATTCTGTTCGTCTCGGAG	161
		Reverse	ATCGATGAGTCGTTCCCGTG	
pparα	AY198322.1	Forward	CCATCCCGACAACGAGTTCC	121
		Reverse	CAGCGACGTGTCTTCTGTCT	

Accession numbers and primers sequences of the genes employed in the RT-qPCR assays

ef-1α elongation factor-1α, *bmal1a* brain and muscle ARNT-like 1a, *pparα* peroxisome proliferator-activated receptor α

was determined by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistics

Data are presented as mean + s.e.m. When necessary, data were log-transformed to fulfil the conditions of normality and homoscedasticity for analysis of variance (ANOVA). Statistical analysis of changes in body mass and locomotor activity was performed using one-way ANOVA followed by the Student–Newman–Keuls (SNK) test, and differences were considered statistically significant at $P < 0.05$. For plasma and tissue metabolites, enzyme activities, and gene expression, statistical analysis was performed using two-way ANOVA with experimental time (acute and chronic) and treatment (control and OEA) as main factors. In those cases where a significant effect was noted for a factor, comparisons were carried out by SNK test. Differences were considered statistically significant at $P < 0.05$.

Results

The chronic effects of OEA on body mass and locomotor activity in goldfish are presented in Fig. 1. OEA treatment impairs the increase in body mass observed in control fish ($P = 0.006$) as shown in Fig. 1a. Fish injected with OEA over 11 days showed a significant reduction in the mean of daily locomotor activity ($P < 0.001$) compared with control fish (Fig. 1b).

Plasma concentration of metabolites and cortisol after acute or chronic treatment with OEA is shown in Fig. 2. OEA administration for 11 days increased plasma concentration of fatty acid ($P = 0.012$) and glucose ($P = 0.032$) (Fig. 2a, d), and decreased triglyceride concentration ($P = 0.046$) compared with the control group (Fig. 2b). Cortisol concentration remained unchanged in all

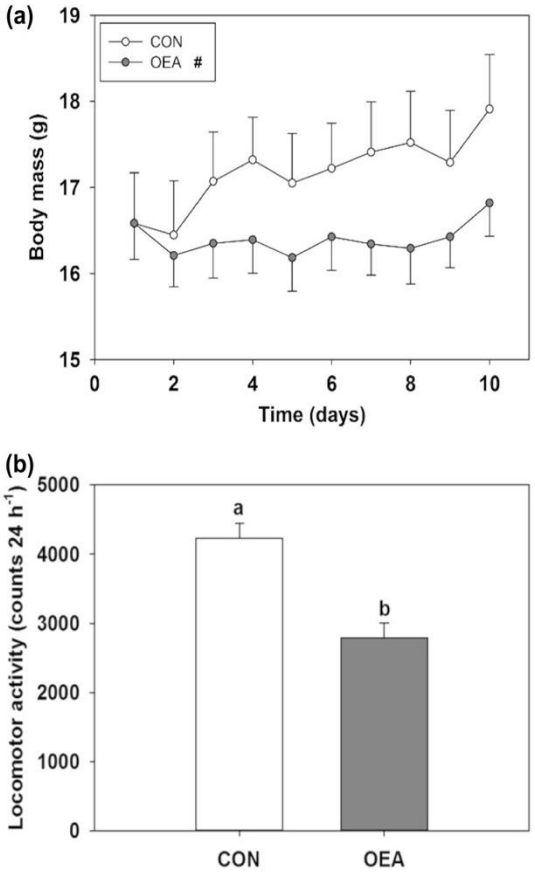


Fig. 1 Body mass and locomotor activity after chronic OEA treatment in goldfish. **a** Body mass profile in fish daily ip injected with vehicle alone (control, CON) or containing OEA (5 μg g^{-1} body mass). Data are expressed as mean ± s.e.m. ($N = 10$ fish per group). *Hash* indicates statistically significant differences between control- and OEA-profile (one-way ANOVA $P < 0.05$, post hoc SNK test $P < 0.05$). **b** Locomotor activity during 24 h after ip administration of control or OEA (5 μg g^{-1} body mass). Data are expressed as mean + s.e.m of 11 days. *Different letters* indicate significant differences between experimental groups (one-way ANOVA $P < 0.05$, post hoc SNK test $P < 0.05$)

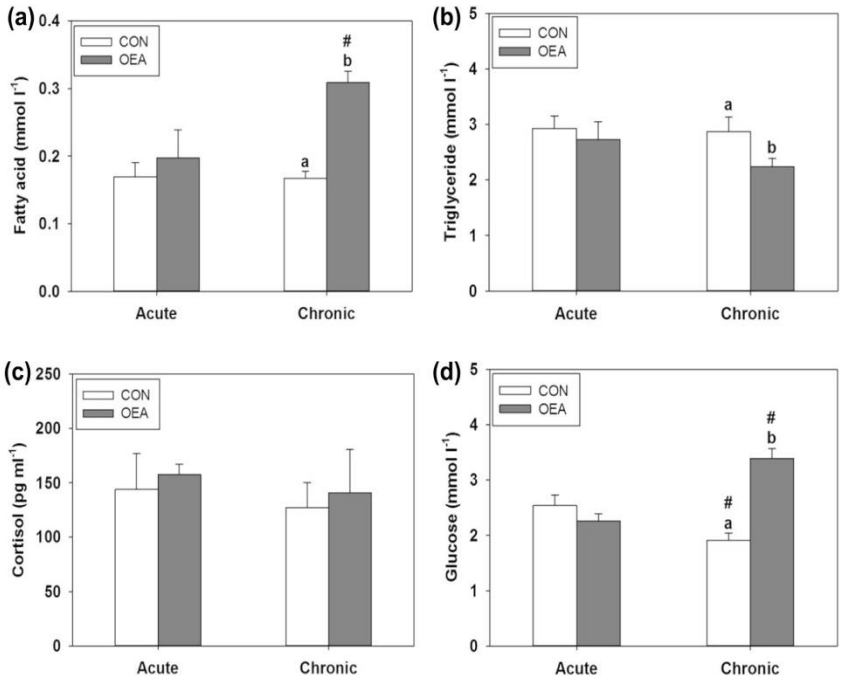


Fig. 2 Effect of OEA on plasma concentration of metabolites and cortisol in goldfish. Fatty acid (**a**), triglyceride (**b**), cortisol (**c**), and glucose (**d**) in plasma after 6 h (acute) or 11 days (chronic) of ip administration of vehicle alone (control, CON) or containing OEA (5 $\mu g\ g^{-1}$ body mass). Each value is expressed as mean + s.e.m. ($N = 6$–8 fish per treatment). *Different letters* indicate significant differences between experimental groups in each experiment, and *hash* indicates significant differences between acute or chronic treatment in the same experimental group (two-way ANOVA $P < 0.05$, post hoc SNK test $P < 0.05$)

treatments (Fig. 2c). No significant differences in concentration of circulating metabolites were observed after acute OEA administration.

Hepatic concentration of metabolites is shown in Fig. 3. The concentration of total lipid (Fig. 3c) and glycogen (Fig. 3e) increased after 6 h of acute OEA injection compared with controls ($P = 0.003$ and $P = 0.009$, respectively). The ip treatment with OEA did not modify hepatic concentration of fatty acid, triglyceride, glucose, and lactate in the acute experiment. OEA chronic treatment did not alter concentrations of metabolites in liver. Levels of total lipid, glucose, and glycogen in controls, and levels of lactate in control and OEA groups showed significant differences between experiments (acute vs. chronic).

The activity of enzymes involved in lipid metabolism assessed in liver is shown in Fig. 4. The activity of ACLY (Fig. 4a) increased after OEA treatment in the acute experiment ($P = 0.012$), but decreased in the chronic experiment ($P < 0.001$), and significant differences between experiments were also observed. The activity of CPT-1 (Fig. 4c) dramatically decreased after OEA treatment in both experiments ($P < 0.001$). No significant changes were noted for FAS and HOAD activities.

The activity of enzymes involved in glucose metabolism assessed in liver is shown in Fig. 5. The activity of HK (Fig. 5a) was lower in the OEA than in control group in the chronic experiment ($P = 0.002$). GK activity (Fig. 5b) decreased after chronic OEA treatment ($P < 0.001$). The activity of PEPCK (Fig. 5f) dramatically decreased after OEA treatment in both experiments ($P < 0.001$). The activity of GPase (Fig. 5g) was higher after OEA treatment in acute experiment ($P < 0.001$). The activity of GSase (Fig. 5h) decreased after chronic OEA treatment ($P = 0.037$). No significant differences due to OEA treatment were observed in the remaining parameters. Significant differences when comparing chronic vs. acute treatment were noted for the activities of HK (increase in control, decrease in treated fish), GK (increase in control fish), PFK (increase in control and treated fish), FBPase (increase in control and treated fish), GSase (decrease in treated fish), G6Pase (increase in treated fish), and G6PDH (increase in control fish).

The effects of peripheral OEA treatment on hepatic expression of *bmal1a* and *pparα* are shown in Fig. 6. The acute OEA treatment did not affect the relative mRNA expression of these genes, whereas the chronic treatment with OEA increased the expression of *bmal1a* (Fig. 6a).

Discussion

OEA treatment impairs the increase in body mass and reduces locomotor activity in goldfish

We demonstrate for the first time in any fish species that chronic treatment with OEA impairs the increase in body mass observed in controls. Despite fish were fed each day 3 h before injection to minimize the effects of OEA in food intake, we cannot discard that at least part of the differences observed in body mass and/or metabolic changes

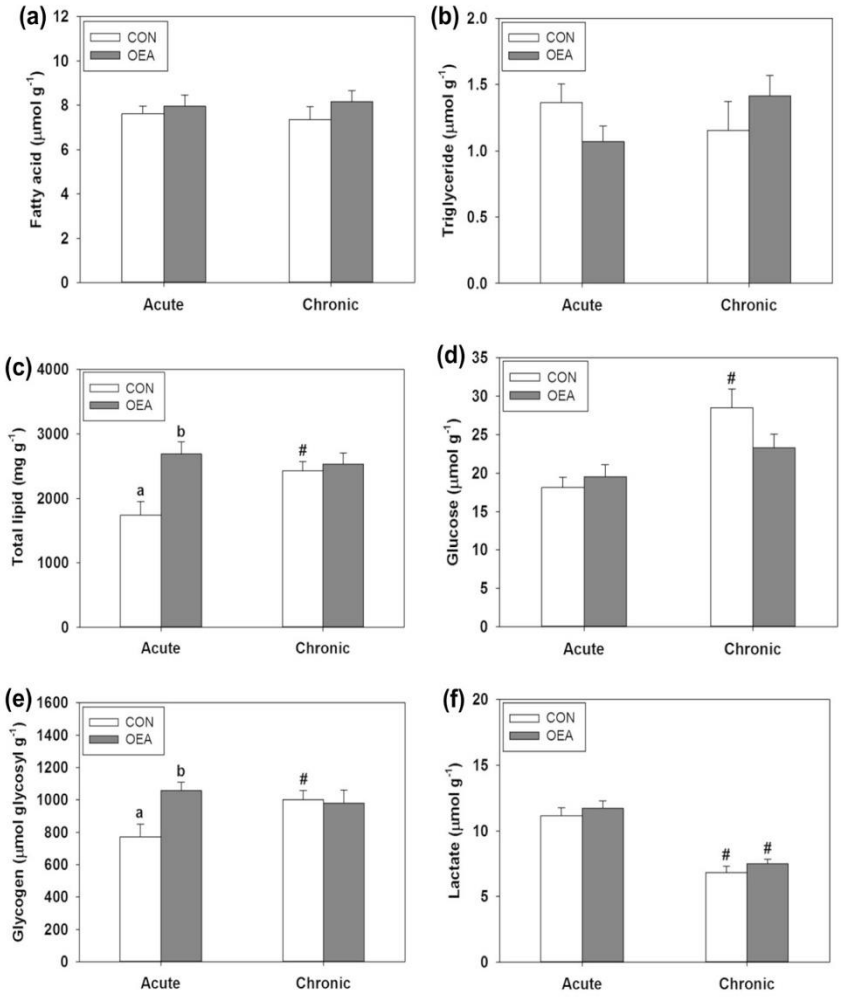


Fig. 3 Effect of OEA on hepatic concentration of metabolites in goldfish. Fatty acid (**a**), triglyceride (**b**), total lipid (**c**), glucose (**d**), glycogen (**e**), and lactate (**f**) in liver after 6 h (acute) or 11 days (chronic) of ip administration of vehicle alone (control, CON) or containing OEA (5 μg g^{-1} body mass). Each value is expressed as mean + s.e.m. ($N = 6$–10 fish per treatment). *Different letters* indicate significant differences between experimental groups in each experiment, and *hash* indicates significant differences between acute or chronic treatment in the same experimental group (two-way ANOVA $P < 0.05$, post hoc SNK test $P < 0.05$)

(see below) between control and OEA-treated fish could be attributed to reduced food intake. This finding is in agreement with that observed in rodents (Rodríguez de Fonseca et al. 2001; Guzmán et al. 2004; Fu et al. 2005; Serrano et al. 2008), and also with the decreased food intake previously described in goldfish after OEA acute treatment (Tinoco et al. 2014). Chronic OEA treatment also resulted in decreased daily locomotor activity in goldfish, confirming previous results in the same species after acute treatment (Tinoco et al. 2014), and in agreement with comparable results observed in mammals (Rodríguez de Fonseca et al. 2001; Proulx et al. 2005). These results support the anorectic action of OEA in goldfish, and reinforce its role as a signal of nutritional status that can regulate feeding behaviour and body mass homeostasis in fish, as in mammals (Piomelli 2013).

We have also assessed changes in plasma cortisol concentration to eliminate any possible stress effect during treatments. No changes were noted between control and treated fish both after acute or chronic OEA treatments, with plasma cortisol concentration being similar to that reported in unstressed goldfish (Valenti et al. 2005; Azpeleta et al. 2010). Similar results have been described in mammals, where corticosterone levels do not change after OEA administration, suggesting that the anorectic effect of OEA cannot be attributed to stress (Rodríguez de Fonseca et al. 2001; Proulx et al. 2005). Therefore, we may suggest that our fish were not stressed by treatment.

OEA treatment in goldfish resulted in decreased liver lipolytic capacity after both acute and chronic OEA treatments, whereas lipogenic capacity increased after acute and decreased after chronic treatment

Chronic OEA treatment induced a clear increase in fatty acid concentration while decreased concentration was noted for triglyceride in plasma. The decrease observed in triglyceride concentration confirms previous results in goldfish (Tinoco et al. 2014) and is comparable with that observed in rodents after chronic OEA treatment (Fu et al.

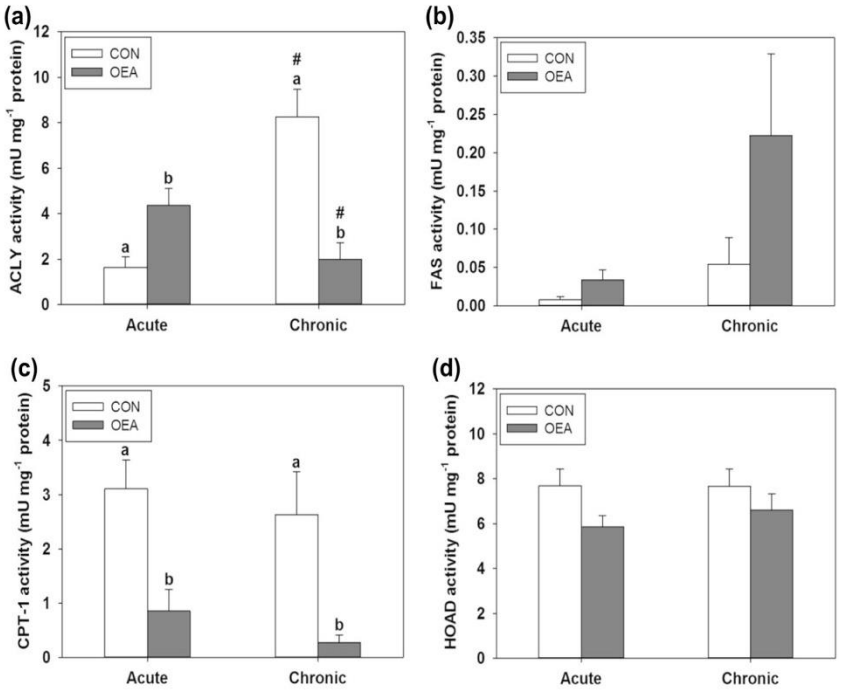


Fig. 4 Effects of OEA on activities of enzymes involved in lipid metabolism in goldfish liver. ACLY (**a**), FAS (**b**), CPT-1 (**c**), and HOAD (**d**) activities after 6 h (acute) or 11 days (chronic) of ip administration of vehicle alone (control, CON) or containing OEA (5 μg g^{-1} body mass). Each value is expressed as mean + s.e.m. ($N = 6$–8 fish per treatment). *Different letters* indicate significant differences between experimental groups in each experiment, and *hash* indicates significant differences between acute or chronic treatment in the same experimental group (two-way ANOVA $P < 0.05$, post hoc SNK test $P < 0.05$)

2005; Yang et al. 2007; Thabuis et al. 2011), whereas the increase in fatty acid concentration is also comparable with the increase observed in rat after acute OEA treatment (Guzmán et al. 2004). These changes could be the consequence of an enhanced lipolysis from lipid stores, but probably not from liver (see below).

This is the first time, as far as we are aware, in which the metabolic effects of OEA have been assessed by changes in the concentration of metabolites and in the activity of enzymes involved in lipid metabolism. In liver metabolites, the only change induced by OEA treatment was the increased concentration of total lipid after acute, but not after chronic treatment. In mammals, OEA treatment decreased triglyceride concentration in liver in rat after acute (Guzmán et al. 2004) but not after chronic (Suárez et al. 2014) treatment, whereas liver fatty acid concentration increased after chronic OEA treatment (Thabuis et al. 2011). The absence of changes in metabolite concentration in this tissue may indicate a rapid transport of metabolites such as fatty acids from liver to plasma.

The acute OEA treatment clearly induced a decrease in the activity of CPT-1 and an increase in the activity of ACLY in liver. These changes would suggest an enhanced lipogenic capacity (ACLY activity) and decreased potential of mitochondrial β-oxidation (CPT-1 activity). These changes are supported by the trends observed in the activity of other hepatic enzymes, such as the lipogenic FAS and the lipolytic HOAD, which despite not showing significant differences displayed the same trends as those of the other enzymes (increased FAS and decreased HOAD activities). There are no available studies in any vertebrate species assessing changes in enzyme activities of lipid metabolism in any tissue after OEA treatment for comparison with our data. The only available study in liver assessed changes in the mRNA abundance of several metabolic enzymes involved in these pathways after acute OEA treatment in rat (Guzmán et al. 2004). The parameters assessed were those involved in fatty acid transport (FABP and FAT/CD36) and mitochondrial activity (UCP2) that increased after OEA treatment in agreement with the enhanced lipid oxidation observed simultaneously in hepatocytes suggesting an enhanced lipolytic potential, i.e. a different response from that observed in the present study in goldfish.

The chronic treatment with OEA induced an apparent decrease in lipogenic capacity based on the decrease observed in ACLY activity whereas the lipolytic capacity was again apparently inhibited based on the decrease observed in the activity of CPT-1. Again, there are no references available describing changes in enzyme activities of lipid metabolism in any tissue after chronic OEA treatment. The available data in rodent liver describe changes in the mRNA abundance of several metabolic enzymes after chronic OEA treatment (Serrano et al. 2008; Thabuis et al. 2011) or OEA-analogue (Decara et al. 2012). However, the evidence obtained is contradictory since OEA treatment increased mRNA abundance of a lipogenic enzyme such as FAS, but decreased mRNA abundance of another lipogenic enzyme such as SCD-1 in rat liver in the Thabuis et al.

J Comp Physiol B (2016) 186:1009–1021

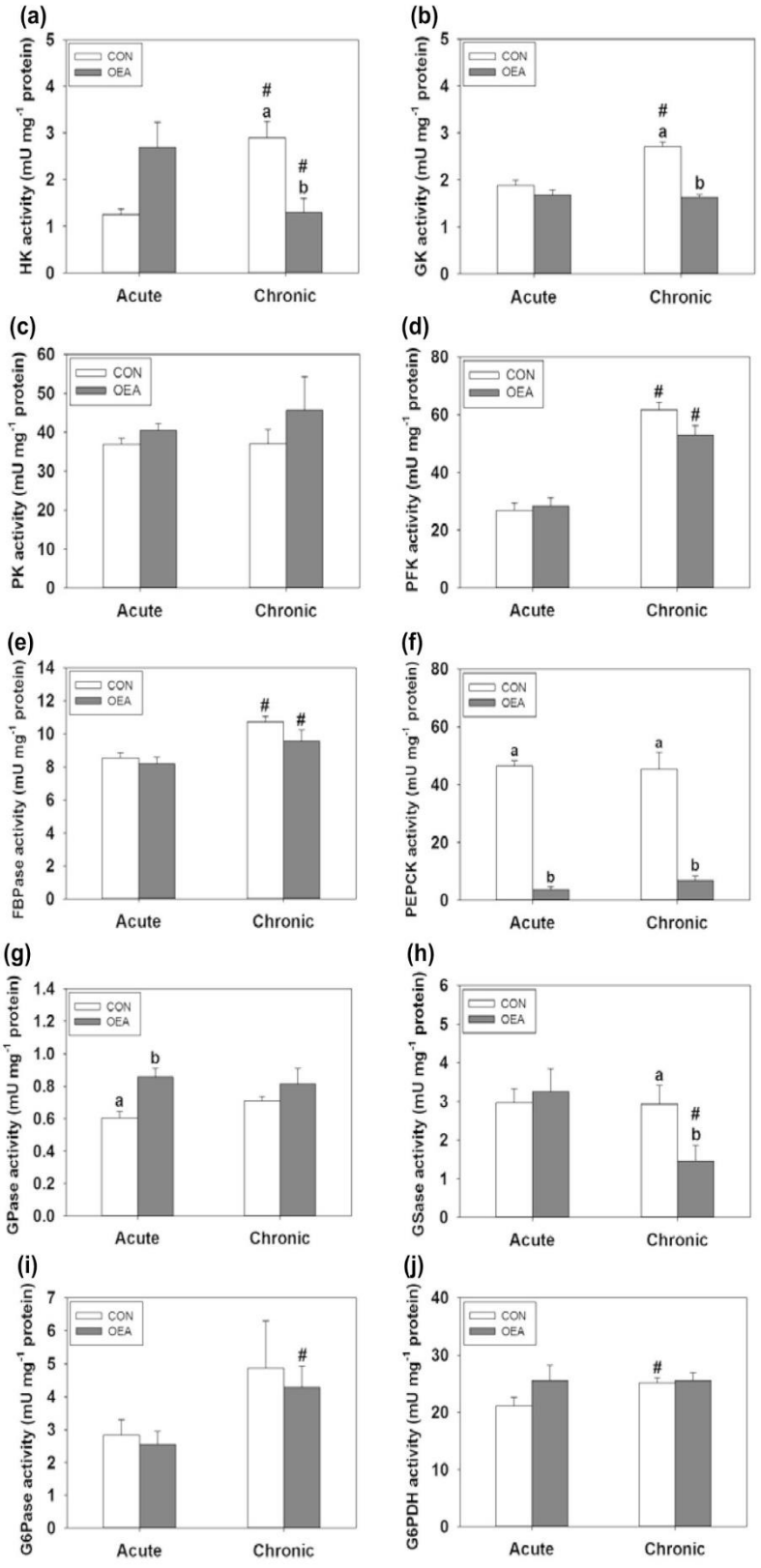


Fig. 5 Effects of OEA on activities of enzymes involved in glucose metabolism in goldfish liver. HK (**a**), GK (**b**), PK (**c**), PFK (**d**), FBPase (**e**), PEPCK (**f**), GPase (**g**), GSase (**h**), G6Pase (**i**), and G6PDH (**j**) activities after 6 h (acute) or 11 days (chronic) of ip administration of vehicle alone (control, CON) or containing OEA (5 μg g^{-1} body mass). Each value is expressed as mean + s.e.m. ($N = 6–8$ fish per treatment). *Different letters* indicate significant differences between experimental groups in each experiment, and *hash* indicates significant differences between acute or chronic treatment in the same experimental group (two-way ANOVA $P < 0.05$, post hoc SNK test $P < 0.05$)

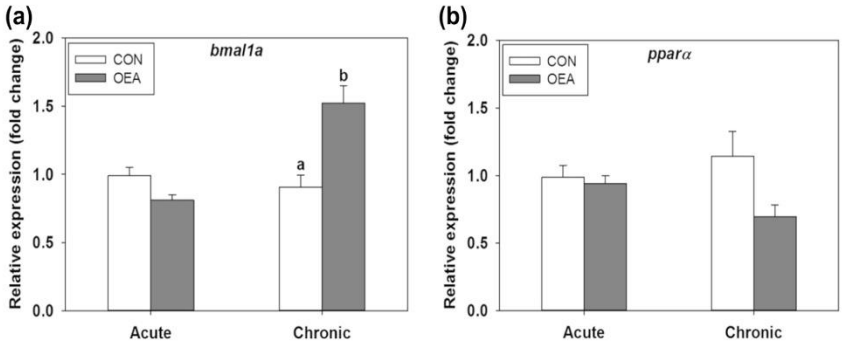


Fig. 6 Effects of OEA on expression of *bmal1a* and *pparα* in goldfish liver. Relative expression of *bmal1a* (**a**) and *pparα* (**b**) after 6 h (acute) or 11 days (chronic) of ip administration of vehicle alone (control, CON) or containing OEA 5 (μg g^{-1} body mass). Data obtained by RT-qPCR are shown as mean + s.e.m. ($N = 7$) in relative units ($2^{-\Delta\Delta Ct}$ method; OEA groups are relativized to their respective control—acute or chronic treatment). *Different letters* indicate significant differences between experimental groups in each experiment (two-way ANOVA $P < 0.05$, post hoc SNK test $P < 0.05$)

(2011) study. In other studies, no changes were noted in the mRNA abundance of lipogenic enzymes in liver (Fu et al. 2005; Serrano et al. 2008). As for lipolytic effects of OEA in liver, the evidence is also contradictory since decreased (Decara et al. 2012) and increased (Guzmán et al. 2004; Fu et al. 2005) mRNA abundance of enzymes involved in lipid metabolism has both been observed. In this way, it is interesting that the OEA-analogue elaidyl-sulfamide enhanced β-oxidation in rat adipose tissue but not in liver (Decara et al. 2012), suggesting a clear dependence on tissue. Thus, the lipolytic effect of OEA after chronic treatment in rodents is consistent in adipose tissue (Suárez et al. 2014) but not in liver, suggesting a clear tissue dependence of OEA effects in mammals. In fish, the only available evidence of OEA effects in liver is the present study, which in general displayed different results from those observed in mammalian adipocytes, but not so different from those observed in mammalian liver.

We did not observe changes in the expression of *pparα* in liver either after OEA acute or chronic treatment. This was a priori unexpected since OEA in several studies in mammalian liver is known to increase *pparα* mRNA abundance after acute (Guzmán et al. 2004) or chronic (Fu et al. 2005) treatment, though decreased *pparα* mRNA abundance was indeed noted in liver of rats treated with an OEA-analogue (Decara et al. 2012). If this transcription factor is not affected by OEA in goldfish liver, this would match with the results obtained since the known metabolic effects of the activation of this transcription factor (enhanced lipolysis) in mammals (Berger and Moller 2002; Liu et al. 2014) would not be taking place in fish. We do not know why this transcription factor is not activated in fish liver, but it suggests a different underlying mechanism in fish compared with the mammalian model. Nevertheless, several studies in mammals also showed that OEA may act through an orphan G-protein coupled receptor (GPR119) and the transient receptor potential vanilloid subtype 1 (TRPV1), instead of PPARα (Romano et al. 2015).

Since we observed that chronic OEA treatment enhanced mRNA abundance of *bmal1a*, at least the decreased lipolytic potential observed in liver of OEA-treated goldfish could be also related to the effects of enhanced *bmal1a* expression, a clock gene with a lipogenic role in rodents. In rat adipocytes, the over-expression of this gene decreases lipolytic and enhances lipogenic capacities (Shimba et al. 2005, 2011), and in mouse hepatocytes, *bmal1* mediates insulin-induced lipogenesis (Zhang et al. 2014). There are no comparable studies available in fish liver though the circadian changes observed in the activity and mRNA abundance of lipogenic and lipolytic enzymes in rainbow trout liver (Hernández-Pérez et al. 2015) are in phase with those of *bmal1* in the same tissue (Hernández-Pérez et al. unpublished). Moreover, in goldfish, food intake increases OEA intestinal content (Tinoco et al. 2014) and *bmal1a* hepatic expression (Gómez-Boronat et al. unpublished data); reinforcing the hypothesis that *bmal1* could mediate some of the OEA lipogenic responses in fish.

CPT-1 is the protein involved in the mitochondrial use of fatty acid, and it has been shown to be clearly inhibited when the levels of a long chain fatty acid like oleate are raised in rainbow trout central and peripheral tissues including liver (Librán-Pérez et al. 2012, 2013a, b, 2015). Since OEA is structurally related to oleate, we cannot eliminate the possibility that besides changes induced in lipid metabolism by activation of transcription factors like PPARα, OEA can induce at least some of its responses through fatty acid sensing mechanisms. In fact, both oleate (Librán-Pérez et al. 2012) and OEA (Tinoco et al. 2014) are anorectic in fish. In this way, the effects of OEA on parameters assessed would also reflect its action at different

levels of fatty acid sensing mechanisms (Soengas 2014). It is interesting in this case that OEA is also known in mammals to induce changes in parameters related to fatty acid sensing, such as enhanced mRNA abundance of the fatty acid translocase FAT/CD36 (Guzmán et al. 2004; Fu et al. 2005; Yang et al. 2007), the fatty acid carrier related to one of the mechanisms involved in fatty acid sensing (Soengas 2014). Therefore, we cannot eliminate the possibility that the effects of OEA may be attributed in part to its interaction with fatty acid sensing systems.

Finally, chronic OEA treatment in mammals has been reported to increase plasma levels of insulin (González-Yanes et al. 2005; Suárez et al. 2014) and adiponectin (Serrano et al. 2011; Suárez et al. 2014), and to reduce those of ghrelin (Serrano et al. 2011), hormones known to induce changes in lipid metabolism in fish tissues including liver (Caruso and Sheridan 2011; Sánchez-Gurmaches et al. 2012; Jönsson 2013; Velasco et al. 2016a, b). If something similar occurred in goldfish, the changes observed in metabolic parameters could be also partially attributed to changes in the levels of these hormones. In fact, acute administration of OEA reduced ghrelin mRNA levels in goldfish intestine (Tinoco et al. 2014). It is particularly interesting that adiponectin is known to induce a decrease in the expression of *pparα* in fish liver (Sánchez-Gurmaches et al. 2012). Considering that we have observed a no significant decrease in mRNA abundance of *pparα* after chronic OEA treatment, the reason of not observing the effects initially expected for OEA might also relate to the interaction with the effects of adiponectin.

OEA treatment decreases glucose use in goldfish liver

There are few references available concerning the impact of OEA on glucose metabolism in mammals. In rat acute (González-Yanes et al. 2005) and chronic (Fu et al. 2005; Serrano et al. 2008; Suárez et al. 2014) treatment with OEA did not affect plasma glucose concentration though the treatment with the OEA-analogue elaidyl-sulfamide induced hyperglycaemia (Decara et al. 2012). Acute OEA treatment decreased glucose uptake in rat adipocytes in one study (González-Yanes et al. 2005) but not in another (Guzmán et al. 2004), whereas in muscle OEA did not alter glucose uptake or oxidation (Guzmán et al. 2004). The only reference available in liver points to decreased glucose oxidation in mice after chronic OEA treatment (Thabuis et al. 2011).

In fish, only our previous study (Tinoco et al. 2014) assessed the impact of acute OEA treatment on plasma glucose concentration with no significant changes in this parameter. In the present study, we observed the same response in plasma after acute OEA treatment though chronic OEA treatment clearly induced hyperglycaemia. This hyperglycaemia is not apparently the result of stress in fish since plasma cortisol concentration was not changed. This is the first time, as far as we are aware, even in mammals, in which a full set of enzyme activities related to glucose metabolism has been assessed in liver in response to the presence of OEA; available studies in mammals elucidated some metabolic rates (Guzmán et al. 2004; González-Yanes et al. 2005; Thabuis et al. 2011) and gene expression (Decara et al. 2012).

The acute OEA treatment induced few changes in liver parameters beyond a clear inhibition of PEPCK activity and increased glycogen concentration. In contrast, the chronic OEA treatment induced several changes in parameters related to hepatic glucose metabolism. First, a clear decrease was noted in the capacity of liver to use exogenous glucose based on the decreased activities of HK and GK. Thus, the liver of OEA-treated fish used less glucose from plasma. This decreased availability of glucose affects the different subsequent uses of glucose within the liver. The clearest effect is that observed in gluconeogenic potential that was inhibited by OEA, after both acute and chronic treatments, based on the clear decrease observed in PEPCK activity. Thus, if the liver is using less glucose, it seems reasonable that the synthesis of glucose is inhibited. Liver PEPCK is an enzyme that is known in fish liver to be directly regulated by hormones like insulin (Polakof et al. 2012). The sharp decrease in its activity suggests a possible endocrine modulation through changes in the levels of insulin or adiponectin induced by OEA treatment, as described above for lipid metabolism. Moreover, since the over-expression of *bmal1* is known to induce decreased mRNA abundance of PEPCK in rat adipocytes (Shimba et al. 2005), the decreased PEPCK activity observed in liver of OEA-treated fish could be also associated, at least in part, with the increased mRNA abundance of *bmal1a* observed simultaneously.

Another clear change observed in the OEA-treated fish was the decreased activity of GSase, i.e. the enzyme involved in glycogen synthesis, which again is reasonable since if less glucose is used the necessity of storing glycogen in this tissue is not so important. The fact that GPase activity and the concentration of glycogen did not change after OEA treatment supports these changes. Other pathways involved in the use of glucose such as the pentose phosphate pathway did not show changes after OEA treatment based on the lack of changes displayed by G6PDH activity.

As commented above, there are no references available with which to compare these data, though the reduced glucose oxidation observed in liver of OEA-treated mice (Thabuis et al. 2011) indirectly supports the changes observed here. Considering the effects of OEA on hepatic glucose metabolism, basically suggesting a reduced glucose

use in liver, this could relate to an increased export capacity of the tissue that would match with the increased glucose concentration observed in plasma simultaneously. However, this was not apparently the case since the activity of the enzyme involved in glucose export capacity from the liver into the plasma, i.e. G6Pase was not altered by OEA. If liver is not increasing glucose export capacity into plasma, the raised glucose concentration in plasma of chronically treated OEA fish might result from a reduced uptake into peripheral tissues, such as adipose and muscle, which is reasonable considering that OEA decreased glucose uptake into rat adipocytes (González-Yanes et al. 2005).

In summary, we demonstrated for the first time in fish that acute or chronic OEA treatment affected lipid and glucose metabolism in liver of goldfish. As for lipid metabolism, the lipolytic capacity in liver decreased after both acute and chronic OEA treatments, whereas lipogenic capacity was increased by acute, but decreased by chronic treatments with OEA. These results are different from those observed to date in mammalian adipose tissue but not so clearly in liver. The differential response could be attributed to different reasons including (among others) the absence of changes in the mRNA abundance of *pparα*, the effect of clock genes, the interaction with fatty acid sensing mechanisms, or the effect of other circulating hormones. As for glucose metabolism, this is the first time the effects of OEA have been assessed in liver, showing after chronic treatment a clear decrease in the capacity of this tissue to use glucose. Considering the central role of the liver in glucose homeostasis in fish (Polakof et al. 2012), this decreased capacity would result in increased plasma availability, in agreement not only with the observed hyperglycaemia in OEA-treated fish but also with the known reduced glucose tolerance reported in mammals (González-Yanes et al. 2005). Together, these results support an important role for OEA in the regulation of liver energy metabolism in fish. Further studies are necessary to fully characterize the underlying mechanisms, the interaction with other regulatory pathways, and its involvement with the metabolic changes associated with circadian activity and the regulation of food intake.

Acknowledgments This study was supported by Spanish Ministerio de Economía y Competitividad (MINECO) research grant (AGL2013-46448-C3-2-R) to M.J.D, and by MINECO and European Fund for Regional Development research grant (AGL2013-46448-C3-1-R and FEDER) to J.L.S. M.G-B. and C.V. were recipients of predoctoral fellowship from MINECO (BES-2014-068103) and Universidade de Vigo, respectively.

References

Astarita G, Rourke BC, Andersen JB, Fu J, Kim JH, Bennett AF, Hicks JW, Piomelli D (2006) Postprandial increase of oleoylethanolamide mobilization in small intestine of the Burmese python (*Python molurus*). Am J Physiol Regul Integr Comp Physiol 290:R1407–R1412

Azari EK, Ramachandran D, Weibel S, Arnold M, Romano A, Gaetani S, Langhans W, Mansouri A (2014) Vagal afferents are not necessary for the satiety effect of the gut lipid messenger oleoylethanolamide. Am J Physiol Regul Integr Comp Physiol 307:R167–R178

Azpeleta C, Martínez-Álvarez RM, Delgado MJ, Isorna E, de Pedro N (2010) Melatonin reduces locomotor activity and circulating cortisol in goldfish. Horm Behav 57:323–329

Berger J, Moller DE (2002) The mechanisms of action of PPARs. Annu Rev Med 53:409–435

Caruso MA, Sheridan MA (2011) New insights into the signaling system and function of insulin in fish. Gen Comp Endocrinol 173:227–247

De Pedro N, Martínez-Álvarez R, Delgado MJ (2006) Acute and chronic leptin reduces food intake and body weight in goldfish (*Carassius auratus*). J Endocrinol 188:513–520

Decara JM, Romero-Cuevas M, Rivera P, Macías-González M, Vida M, Pavón FJ, Serrano A, Cano C, Fresno N, Pérez-Fernández R, Rodríguez de Fonseca F, Suárez J (2012) Elaidyl-sulfamide, an oleoylethanolamide-modelled PPARα agonist, reduces body weight gain and plasma cholesterol in rats. Dis Models Mech 5:660–670

Fu J, Oveisi F, Gaetani S, Lin E, Piomelli D (2005) Oleoylethanolamide, and endogenous PPAR-α agonist, lowers body weight and hyperlipidemia in obese rats. Neuropharmacology 48:1147–1153

González-Yanes C, Serrano A, Bermúdez-Silva FJ, Hernández-Domínguez M, Páez-Ochoa MA, Rodríguez de Fonseca F, Sánchez-Margalet V (2005) Oleoylethanolamide impairs glucose tolerance and inhibits insulin-stimulated glucose uptake in rat adipocytes through p38 and JNK MAPK pathways. Am J Physiol Endocrinol Metab 289:E923–E929

Guzmán M, Lo Verme J, Fu J, Oveisi F, Blázquez C, Piomelli D (2004) Oleoylethanolamide stimulates lipolysis by activating the nuclear receptor peroxisome proliferator-activated receptor α (PPAR-α). J Biol Chem 279:27849–27854

Hernández-Pérez J, Míguez JM, Librán-Pérez M, Otero-Rodiño C, Naderi F, Soengas JL, López-Patiño MA (2015) Daily rhythms in activity and mRNA abundance of enzymes involved in glucose and lipid metabolism in liver of rainbow trout, *Oncorhynchus mykiss*. Influence of light and food availability. Chronobiol Int 32:1391–1408

Jönsson E (2013) The role of ghrelin in energy balance regulation in fish. Gen Comp Endocrinol 187:79–85

Keppler D, Decker K (1974) Glycogen determination with amyloglucosidase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Academic Press, New York, pp 1127–1131

Librán-Pérez M, Polakof S, López-Patiño MA, Míguez JM, Soengas JL (2012) Evidence of a metabolic fatty-acid sensing system in the hypothalamus and Brockmann bodies of rainbow trout: implications in food intake regulation. Am J Physiol Regul Integr Comp Physiol 302:R1340–R1350

Librán-Pérez M, Figueiredo-Silva AC, Panserat S, Geurden I, Míguez JM, Polakof S, Soengas JL (2013a) Response of hepatic lipid and glucose metabolism to a mixture or single fatty acids: possible presence of fatty acid-sensing mechanisms. Comp Biochem Physiol A 164:241–248

Librán-Pérez M, López-Patiño MA, Míguez JM, Soengas JL (2013b) In vitro response of putative fatty acid-sensing systems in rainbow trout liver to increased levels of oleate or octanoate. Comp Biochem Physiol A 165:288–294

Librán-Pérez M, Geurden I, Dias K, Corraze G, Panserat S, Soengas JL (2015) Feeding rainbow trout with a lipid-enriched diet: effects on fatty acid sensing, regulation of food intake and cellular signaling pathways. J Exp Biol 218:2610–2619

Liu S, Alexander RK, Lee CH (2014) Lipid metabolites as metabolic messengers in inter-organ communication. Trends Endocrinol Metab 25:356–363

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408

Piomelli D (2013) A fatty gut feeling. Trends Endocrinol Metab 7:332–341

Polakof S, Míguez JM, Moon TW, Soengas JL (2007a) Evidence for the presence of a glucosensor in hypothalamus, hindbrain, and Brockmann bodies of rainbow trout. Am J Physiol Regul Integr Comp Physiol 292:R1657–R1666

Polakof S, Míguez JM, Soengas JL (2007b) In vitro evidences for glucosensing capacity and mechanisms in hypothalamus, hindbrain, and Brockmann bodies of rainbow trout. Am J Physiol Regul Integr Comp Physiol 293:R1410–R1420

Polakof S, Míguez JM, Soengas JL (2008a) Changes in food intake and glucosensing function of hypothalamus and hindbrain in rainbow trout subjected to hyperglycemic or hypoglycemic conditions. J Comp Physiol A 194:829–839

Polakof S, Míguez JM, Soengas JL (2008b) Dietary carbohydrates induce changes in glucosensing capacity and food intake in rainbow trout. Am J Physiol Regul Integr Comp Physiol 295:R478–R489

Polakof S, Panserat S, Plagnes-Juan E, Soengas JL (2008c) Altered dietary carbohydrates significantly affect gene expression of the major glucosensing components in Brockmann bodies and hypothalamus of rainbow trout. Am J Physiol Regul Integr Comp Physiol 295:R1077–R1088

Polakof S, Panserat S, Soengas JL, Moon TW (2012) Glucose metabolism in fish: a review. J Comp Physiol B 182:1015–1045

Proulx K, Cota D, Castaneda TR, Tschop MH, D'Alessio DA, Tso P, Woods SC, Seeley RJ (2005) Mechanisms of oleoylethanolamide-induced changes in feeding behavior and motor activity. Am J Physiol Regul Integr Comp Physiol 289:R729–R737

Rodríguez de Fonseca F, Navarro M, Gómez R, Escuredo L, Nava F, Fu J, Murillo-Rodríguez E, Giuffrida A, Lo Verme J, Gaetani S (2001) An anorexic lipid mediator regulated by feeding. Nature 414:209–212

Romano A, Soares Potes C, Tempesta B, Cassano T, Cuomo V, Lutz T, Gaetani S (2013) Hindbrain noradrenergic input to the hypothalamic PVN mediates the activation of oxytocinergic neurons induced by the satiety factor oleoylethanolamide. Am J Physiol Endocrinol Metab 305:E1266–E1273

Romano A, Tempesta B, Provensi G, Passani MB, Gaetani S (2015) Central mechanisms mediating the hypophagic effects of oleoylethanolamide and *N*-acylphosphatidylethanolamines: different lipid signals? Front Pharmacol 6:137

Sánchez-Bretaño A, Callejo M, Montero M, Alonso-Gómez AL, Delgado MJ, Isorna E (2016) Performing a hepatic timing signal: glucocorticoids induce gper1a and gper1b expression and repress gclock1a and gbmal1a in the liver of goldfish. J Comp Physiol B 186:73–82

Sánchez-Gurmaches J, Cruz-Garcia L, Gutiérrez J, Navarro I (2012) Adiponectin effects and gene expression in rainbow trout: an in vivo and in vitro approach. J Exp Biol 215:1373–1383

Serrano A, Del Arco I, Pavón FJ, Macías M, Perez-Valero V, Rodríguez de Fonseca F (2008) The cannabinoid CB1 receptor antagonist SR141716A (Rimonabant) enhances the metabolic benefits of long-term treatment with oleoylethanolamide in Zucker rats. Neuropharmacology 54:226–234

Serrano A, Pavón FJ, Tovar S, Casanueva F, Señarís R, Diéguez C, Rodríguez de Fonseca F (2011) Oleoylethanolamide: effects on hypothalamic transmitters and gut peptides regulating food intake. Neuropharmacology 60:593–601

Shimba S, Ishii N, Ohta Y, Ohno T, Watabe Y, Hayashi M, Wada T, Aoyagi T, Tezuka M (2005) Brain and muscle Arnt-like protein-1 (BMAL1) a component of the molecular clock, regulates adipogenesis. Proc Natl Acad Sci USA 102:12071–12076

Shimba S, Ogawa T, Hitosugi S, Ichihashi Y, Nakadaira Y, Kobayashi M, Tezuka M, Kosuge Y, Ishige K, Ito Y, Komiyama K, Okamatsu-Ogura Y, Kimura K, Saito M (2011) Deficient of a clock gene, brain and muscle Arnt-Like Protein-1 (BMAL1), induces dyslipidemia and ectopic fat formation. PLoS One 6:e325231

Soengas JL (2014) Contribution of glucose- and fatty acid sensing systems to the regulation of food intake in fish. A review. Gen Comp Endocrinol 205:36–48

Suárez J, Rivera P, Arrabal S, Crespillo A, Serrano A, Baixeras E, Pavón FJ, Cifuentes M, Nogueiras R, Ballesteros J, Diéguez C, Rodríguez de Fonseca F (2014) Oleoylethanolamide enhances β-adrenergic-mediated thermogenesis and white-to-brown adipocyte phenotype in epididymal white adipose tissue in rat. Dis Models Mech 7:129–141

Thabuis C, Destaillats F, Lambert DM, Muccioli GG, Maillot M, Harach T, Tissot-Favre D, Martin J-C (2011) Lipid transport function is the main target of oral oleoylethanolamide to reduce adiposity in high-fat-fed-mice. J Lipid Res 52:1373–1382

Tinoco AB, Armirotti A, Isorna E, Delgado MJ, Piomelli D, De Pedro N (2014) Role of oleoylethanolamide as a feeding regulator in goldfish. J Exp Biol 217:2761–2769

Valenti M, Cottone E, Martínez R, De Pedro N, Rubio M, Viveros MP, Franzoni MF, Delgado MJ, Di Marzo V (2005) The endocannabinoid system in the brain of *Carassius auratus* and its possible role in the control of food intake. J Neurochem 95:662–672

Velasco C, Otero-Rodiño C, Librán-Pérez M, López-Patiño MA, Míguez JM, Cerdá-Reverter JM, Soengas JL (2016a) Ghrelin modulates hypothalamic fatty acid sensing and control of food intake in rainbow trout. J Endocrinol 228:25–37

Velasco C, Librán-Pérez M, Otero-Rodiño C, López-Patiño MA, Míguez JM, Soengas JL (2016b) Intracerebroventricular ghrelin treatment affects lipid metabolism in liver of rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol 228:33–39

Yang Y, Chen M, Georgeson KE, Harmon CM (2007) Mechanism of oleoylethanolamide on fatty acid uptake in small intestine after food intake and body weight reduction. Am J Physiol Regul Integr Comp Physiol 292:R235–R241

Zhang D, Tong X, Arthurs B, Guha A, Rui L, Kamath A, Inoki K, Yin L (2014) Liver clock protein BMAL1 promotes de novo lipogenesis through insulin-mTORC2-AKT signaling. J Biol Chem 289:25925–25935

Hormones and Behavior xxx (xxxx) xxx-xxx

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

First evidence on the role of palmitoylethanolamide in energy homeostasis in fish

Miguel Gómez-Boronat[a], Esther Isorna[a], Marta Conde-Sieira[b], María J. Delgado[a], José L. Soengas[b], Nuria de Pedro[a*]

[a] Departamento de Genética, Fisiología y Microbiología, Unidad Docente de Fisiología Animal, Facultad de Biología, Universidad Complutense de Madrid, Madrid, Spain
[b] Laboratorio de Fisioloxía Animal, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía and Centro de Investigación Mariña, Universidade de Vigo, Vigo, Spain

ARTICLE INFO

Keywords:
PEA
Food intake
Body mass
Lipid metabolism
Glucose metabolism
Liver
PPAR
Rev-ERB
Clock genes

ABSTRACT

In this study we aimed to elucidate, for the first time in fish, whether palmitoylethanolamide (PEA) is involved in the regulation of energy homeostasis, using goldfish (Carassius auratus) as a model. Therefore, we assessed in different experiments the effects of acute or chronic intraperitoneal treatment with PEA (20 μg.g-1 body weight) on parameters related to food intake and its regulatory mechanisms, body weight, locomotor activity, liver glucose and lipid metabolism, as well as the possible involvement of transcription factors and clock genes on metabolic changes in the liver. PEA treatment induced a decrease in food intake comparable to that observed in mammals. This, anorectic effect of PEA in goldfish could be mediated through interactions with leptin and NPY, as PEA treatment increased hepatic expression of leptin and reduced hypothalamic expression of npy. PEA treatment also reduced weight gain, growth rate and locomotor activity after chronic treatment.In liver metabolism, PEA enhanced the use of glucose not only to be used in the tissue, but probably to be also exported to be used in other tissues. Lipid metabolism was scarcely affected by PEA treatment, with lipogenic effect, in contrast with the mammalian model. Different mechanisms must be involved in mediating such response and the present results provide evidence for the possible involvement of other transcription factors such as REV-ERBα or clock genes like bmal1. As a whole, the present study displayed a global picture of the action of PEA in several compartments related to energy homeostasis, supporting an important regulatory role for this NAE in fish.

1. Introduction

Energy homeostasis involves the coordinated homeostatic regulation of food intake (energy inflow) and energy expenditure (energy outflow). The vertebrate brain, particularly the hypothalamus, plays a central role in regulating energy homeostasis by integrating a number of signals of nervous, metabolic, and endocrine nature that transmit information about energy balance, as demonstrated in mammals (Heisler and Lam, 2017; Rui, 2013) and fish (Delgado et al., 2017; Rønnestad et al., 2017; Soengas et al., 2018; Volkoff, 2016). The N-acylethanolamines (NAEs) are a family of endogenous lipid signaling molecules synthesized in the gastrointestinal tract that have been suggested as important peripheral regulators of energy homeostasis in vertebrates (Hansen, 2014; Kleberg et al., 2014; Romano et al., 2015). The most studied NAE is oleoylethanolamide (OEA), with few information available on the role of other NAEs, such as palmitoylethanolamide (PEA), on the regulation of feeding and metabolism.

PEA is apparently involved in the regulation of food intake in rats (Mattace Raso et al., 2014b), in agreement with postprandial increases in intestine PEA levels observed in different vertebrates, including mammals (Petersen et al., 2006), reptiles (Astarita et al., 2006), and fish (Gómez-Boronat et al., 2019), as well as in the decrease in PEA intestinal content observed in response to dietary fat intake (Diep et al., 2011; Leishman et al., 2016). Accordingly, the anorectic effect of PEA has been demonstrated in rat after acute injection (Rodríguez de Fonseca et al., 2001) or chronic treatment in ovariectomy-induced model of obesity (Mattace Raso et al., 2014a). In fish, there is no evidence for such a role for PEA with the only available studies describing anorectic effects of other NAE, the OEA, in goldfish (Gómez-Boronat et al., 2016; Tinoco et al., 2014).

There is few evidence suggesting that PEA can be also involved in the control of energy expenditure through changes in peripheral energy metabolism (Matias et al., 2007), although a relationship is likely based on the decrease observed in circulating PEA levels in mammals under hyperglycemic conditions (Matias et al., 2007) or after feeding a high-fat diet (Leishman et al., 2016). Accordingly, chronic treatment with PEA in rats reduced body weight and fat mass, and increased β-oxidative capacity in white adipose tissue (Mattace Raso et al., 2014b). Regarding effects of NAEs on liver energy metabolism in vertebrates, some effects of OEA has been described in mammals (González-Yanes et al., 2005; Thabuis et al., 2011) and in fish (Gómez-Boronat et al., 2016), but there is no available evidences on PEA effects to date. As for the possible involvement of PEA in another component of energy expenditure, such as locomotor activity, a recent study in

*Corresponding author at: Departamento de Genética, Fisiología y Microbiología, Unidad Docente de Fisiología Animal, Facultad de Biología, Universidad Complutense de Madrid, Madrid, Spain.
E-mail addresses: ndepedro@bio.ucm.es (N. de Pedro).

https://doi.org/xx.xxxx/j.yhbeh.xxxx.xx.xxx
Received xxx; Received in revised form xxx; Accepted xxx

mice has shown a significant reduction in locomotor activity after PEA treatment (Zambrana-Infantes et al., 2018). There is no evidence available in other vertebrate species, though a decrease is known to occur after OEA treatment both in goldfish (Gómez-Boronat et al., 2016; Tinoco et al., 2014) and mammals (Proulx et al., 2005; Rodríguez de Fonseca et al., 2001). PEA and OEA are endogenous ligands of the transcription factor peroxisome proliferator-activated receptor-α (PPARα; Fu et al., 2003; Hansen and Vana, 2018). Evidences in mammals highlight the importance of PPARs in metabolic regulation (Adamovich et al., 2015). These transcription factors activate or suppress the expression of multiple genes involved in lipid and glucose homeostasis (Grygiel-Górniak, 2014; Pawlak et al., 2015). Moreover, PPARα play an important role in supporting circadian rhythmicity (regulates *bmal1* and *rev-erba* expression), and it is also regulated by circadian gene clocks, CLOCK:BMAL1 (Albrecht and Ripperger, 2018; Ribas-Latre and Eckel-Mahan, 2016). For its part in fish, there are some studies in which daily rhythms of hepatic expression of *ppara*, *bmal1a*, and *rev-erba* were found in goldfish, with similar profiles to that described in the clock circuitry of mammals (Gómez-Boronat et al., 2019). Furthermore, OEA increased hepatic expression of *bmal1a* in the same species, probably by activating the nuclear receptor PPARα (Gómez-Boronat et al., 2016). No available studies assessed the putative effects of PEA on PPARs expression and action.

The main objective of this study was to elucidate whether PEA is involved in the regulation of energy homeostasis in fish, using goldfish (*Carassius auratus*) as a model for which we previously demonstrated effects of OEA on food intake, body weight, locomotor activity, and liver energy metabolism (Gómez-Boronat et al., 2016; Tinoco et al., 2014). Therefore, we assessed the effects of acute or chronic intraperitoneal administration of PEA on food intake, body weight and mRNA abundance of some well-known feeding regulators in hypothalamus such as neuropeptide Y (*npy*), pre-proorexin (*hcrt*), pro-opio melanocortin (*pomc*), cocaine- and amphetamine-regulated transcript (*cartpt*), and leptin aI (*lep aI*) in liver. We also assessed the effects of PEA treatment on energy expenditure through changes in locomotor activity, as well as changes in liver energy metabolism through assessment of metabolite levels and activities and mRNA abundance of enzymes involved in glucose and lipid metabolism. Finally, we also assessed the mRNA abundance of the transcription factors peroxisome proliferator-activated receptor-α (*ppara*), γ (*pparg*) rev-erb-α (*nr1d1*), and β b (*nr1d2 b*) in order to study their putative involvement in PEA regulatory action on energy metabolism, while the relationship with the hepatic circadian circuitry was examined analyzing the mRNA abundance of the hepatic clock genes period (*per*), circadian locomotor output cycles kaput 1a (*clock1a*), and *bmal1a*.

2. Material and methods

2.1. Fish

Goldfish were obtained from a local commercial supplier (ICA, Madrid, Spain). Fish were housed in 60 l aquaria with filtered and aerated freshwater (21 ± 1 °C) under a 12 h light and 12 h darkness (12L:12D) photoperiod (lights on at 8 a.m.). Fish were fed daily at 10 a.m. with commercial dry pellets (1% of body weight, bw; 32.1% crude protein, 5% crude fat, 1.9% crude fibre, 6.8% crude ash, 5.1% water, and 49.1% nitrogen free extract; Sera Pond Biogran, Heinsberg, Germany). Fish were maintained under these conditions for one month before experimental procedures. The aquaria walls were covered with an opaque paper to minimize external interference during the experiments. The experiments described below comply with the Guidelines of the European Union Council (EU 63/2010) and the Spanish Government (RD 53/2013) concerning the protection of animals used for scientific purposes, and were approved by the Animal Experimentation Committee of Complutense University (O.H.-UCM-25-2014) and the Community of Madrid (PROEX 107/14).

2.2. PEA administration

PEA (Sigma-Aldrich, Madrid, Spain) was dissolved in a vehicle consisting of 5% Tween 80 (Sigma-Aldrich), 5% polyethylene glycol (Sigma-Aldrich) and 90% teleost saline (20 mg Na2CO3/100 ml of 0.6% NaCl). Fish were anaesthetized in water containing buffered tricaine methanesulfonate (MS-222, 0.18 g/l; Sigma-Aldrich), weighed, and injected. The intraperitoneal (i.p.) injections were performed using 1 ml syringes and 0.3 mm Microlance needles (Lab-Center, Madrid, Spain), close to the ventral midline and behind the pelvic fins (Gómez-Boronat et al., 2016; Tinoco et al., 2014). Fish were i.p. injected with 10 µl.g-1 of vehicle alone (Control group) or containing PEA (20 µg.g-1 bw). The doses of PEA was chosen based on previous experiments in rats (Rodríguez de Fonseca et al., 2001). No significant effects were observed due to vehicle alone (results not shown). After i.p. injections, fish were transferred to the anaesthetic-free water, where swimming activity and equilibrium were recovered within 1-2 min.

2.3. Experimental designs

2.3.1. Effects of PEA on food intake and mRNA abundance of food intake regulators

A first set of fish (16 ± 4 g bw) was divided into two groups (n = 10 fish/group) and i.p. injected at the scheduled feeding time (10 a.m.) in 24-h fasted fish with vehicle alone or containing PEA (20 µg.g-1 bw), as described above. Then, each fish was individually placed in 5 l aquaria and food intake was quantified at 2, 6, and 8 hours post-injection as previously described (de Pedro et al., 2006; Tinoco et al., 2014). Briefly, pre-weighted dry food was supplied in excess (3% bw) at 10 min post-injection, and any remaining food was collected at 2, 6, and 8 h post-injection and dried at 55 °C for 24 h. Food intake was calculated as: FI = *Wi* – (*Wf* x *f*), where *Wi* and *Wf* are the initial and the remaining dry food weight, respectively, and *f* is the dilution factor (de Pedro et al., 2006).

To investigate the acute effects of PEA on mRNA abundance of food intake regulators, 20 goldfish (17 ± 2 g bw) were i.p. injected with vehicle alone (n = 10) or containing PEA (20 µg.g-1 bw; n = 10) at 10 a.m., and subsequently fed as described above. At 6 h post-injection, fish were sacrificed by anaesthetic overdose (MS-222) followed by spinal cord section. The hypothalamus and liver were quickly collected, frozen in liquid nitrogen, and stored at -80 °C until analysis of mRNA abundance of food intake regulators (*npy*, *pomc*, *hcrt*, *cartpt-I*, and *cartpt-II*, in the hypothalamus, and *lep aI* in the liver).

2.3.2. Effects of PEA on body weight, locomotor activity, metabolism, and hepatic gene expression

Two groups of goldfish (12 ± 2 g bw, n = 12 fish/group) were daily i.p. injected during 10 days with either vehicle alone or containing PEA (20 µg.g-1 bw) as described above. Fish were daily fed at scheduled (10 a.m., 1% bw) and injected at 3-h post-feeding (1 p.m.). Body weight, length, and locomotor activity were recorded throughout the experiment. After the last day of injections, fish were fasted for 48 h and at 1 p.m. (i.e. 48 h post-injection) blood was collected from the caudal vein of anaesthetized animals and plasma was obtained after blood centrifugation (10 min at 2,300 g, 4 °C) and stored at -80 °C until assessment of fatty acid, triglyceride, and glucose. Then, liver was sampled and stored frozen until assessment of metabolite levels (fatty acid, triglyceride, total lipid, glucose, lactate, and glycogen), activity of enzymes involved in lipid and glucose metabolism, and mRNA abundance of metabolic genes (phosphoenolpyruvate carboxykinase 2a, *pck2a*; glucose facilitative carrier type 2, *slc2a2*; and sodium-glucose linked transporter, *slc5a1*), transcription factors (*ppara*, *pparg*, *nr1d1*, and *nr1d2 b*), and clock genes (*per1a*, *per1b*, *per2a*, *per3*, *clock1a*, and *bmal1a*).

To analyze whether the effects of PEA on metabolism and hepatic gene expression are at short-time, an acute experiment was carried

out. For that purpose, 24-h fasted goldfish (22 ± 3 g bw; n = 12 fish/group) were i.p. injected with either vehicle or containing PEA at the scheduled feeding time (10 a.m.) and at 6 h post-injection fish were sampled as described above in the chronic experiment.

2.4. *Analytical techniques*

2.4.1. *Biometric parameters*

Weight increase (bw gain), specific growth rate (SGR), and nutritional index (NI, or condition factor) were determined in the chronic PEA treatment as previously described (de Pedro et al., 2006). Body weight gain per day was calculated as the percentage of bw (g) respect to the initial bw. SGR was calculated as follows: SGR = [(ln *Wf* - ln *Wi*) / *t*] x 100, where *Wi* and *Wf* are initial and final bw, respectively, and *t* is the time interval (in days) between *Wi* and *Wf* measurements. The NI was determined the first and the last day of the experiment as NI = [bw (g) / length (cm)3] x 100.

2.4.2. *Locomotor activity registration*

Daily locomotor activity was recorded during the chronic PEA treatment until the sampling day as previously described (Azpeleta et al., 2010; Gómez-Boronat et al., 2016). Briefly, six infrared photocells (Omron Corporation, E3S-AD12, Japan) were fixed on the walls of each aquarium wall. Two photocells were located below the automatic feeder (registering the locomotor activity related to feeding behavior), while the remaining four photocells were placed at a height of 3-9 cm above the bottom in each aquaria wall (registering the locomotor activity related to general movements). Each photocell continuously emitted an infrared light beam which was interrupted each time fish swam in that zone, generating an output signal. The number of light beam interruptions was automatically registered every 10 min by a computer with specific software (Micronec, Spain). Food anticipatory activity (FAA) was determined as the number of light beam interruptions during the 3-h period prior to daily food delivery (10 a.m.).

2.4.3. *Plasma and tissue metabolites*

Plasma concentration of fatty acid, triglyceride, and glucose was determined enzymatically using commercial kits (Wako Chemicals, Neuss, Germany, for fatty acid; Spinreact, Barcelona, Spain, for triglyceride and glucose) adapted to microplates. Samples to quantify liver metabolites were homogenized by ultrasonic disruption in 7.5 vols of ice-cooled 0.6 N perchloric acid and neutralized with 7.5 vols of ice-cooled 1 N potassium bicarbonate. The homogenate was centrifuged (4 min at 13,500 g, 4 °C) and the supernatant assayed for tissue metabolites. Hepatic concentration of fatty acid, total lipid, triglyceride, glucose, and lactate was determined enzymatically using commercial kits as described above for plasma samples. Liver glycogen was assessed using the method of Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) and tissue glucose concentration was determined with a commercial kit (Biomérieux).

2.4.4. *Liver enzymatic activities*

Liver samples were homogenized by ultrasonic disruption in 9 vols of ice-cooled-buffer consisting of 50 mM imidazole (pH 7.0), 5 mM EDTA, 5 mM EGTA, 15 mM β-mercaptoethanol, 100 mM potassium fluoride, and a protease inhibitory cocktail (Sigma-Aldrich). The homogenate was centrifuged (10 min at 900 g, 4 °C) and the supernatant immediately used for enzyme activities using a microplate reader INFINITE 200 Pro (Tecan, Männedorf, Switzerland) and 96-well microplates. The reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm and, in the case of CPT-1 activity, the increase of 5,5′-dithiobis(2-nitrobenzoic acid)-CoA (DTNB-CoA) complex at 412 nm. The reactions were started by the addition of supernatant (10-50 µl) at a pre-established protein concentration, omitting the substrate in the control wells (final volume 180-295 µl), and allowing the reactions to proceed at 37°C for pre-established times (3-25 min). Enzyme activities are expressed in terms of mg protein, which was assayed using 96-well microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma-Aldrich) as standard. ATP-citrate lyase (ACLY, *EC* 4.1.3.8), fatty acid synthase (FAS, *EC* 2.3.1.85), 3-hydroxyacyl-CoA dehydrogenase (HOAD, EC 1.1.1.35), carnitine palmitoyltransferase-1 (CPT-1, *EC* 2.3.1.21), hexokinase (HK, *EC* 2.7.1.1), glucokinase (GK, *EC* 2.7.1.2), pyruvate kinase (PK, *EC* 2.7.1.40), 6-phosphofructo 1-kinase (PFK, *EC* 2.7.1.11), fructose 1,6-bisphosphatase (FBPase, *EC* 3.1.3.11), phospho(enol)pyruvate carboxykinase (PEPCK, *EC* 4.1.1.32), glycogen synthase (GSase, *EC* 2.4.1.11), glycogen phosphorylase (GPase, *EC* 2.4.1.1), glucose 6-phosphatase (G6Pase, *EC* 3.1.3.9), and glucose 6-phosphate dehydrogenase (G6PDH, *EC* 1.1.1.49) activities were assessed as previously described (Gómez-Boronat et al., 2016; Librán-Pérez et al., 2013a, 2013b, 2012; Polakof et al., 2008a, 2008b, 2008c, 2007a, 2007b). PFK activity was determined at low (0.1 mM) and high (5 mM) fructose 6-phosphate concentrations (omitted for controls), and an activity ratio was calculated as the activity at low fructose 6-phosphate/high fructose 6-phosphate concentrations. GPase active form activity was measured with 10 mM caffeine present, and GPase total activity was estimated without caffeine; the ratio of GPase activities with and without caffeine multiplied by 100 represents the percentage of total GPase (a + b) in the active form (% GPase active).

2.4.5. *Assessment of mRNA abundance*

Real time quantitative PCR (RT-qPCR) was performed as previously described (Gómez-Boronat et al., 2018). Briefly, total RNA from hypothalamus and liver were isolated using TRI® Reagent (Sigma-Aldrich) and treated with RQ1 RNase-Free DNase (Promega, Madison, USA) according to the manufacturer's instructions. Then, 0.5 µg of total RNA was reverse transcribed into cDNA in a 25 µl reaction volume using random primers (Invitrogen, Carlsbad, USA), RNase inhibitor (Promega) and SuperScript II Reverse Transcriptase (Invitrogen). The reverse transcription reaction consisted of an initial step at 25 °C for 10 min, an extension at 42 °C for 50 min, and a denaturalization step at 70 °C for 15 min. RT-qPCRs were carried out by duplicate in a CFX96™ Real-Time System (Bio-Rad Laboratories, Hercules, USA), using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) into a 96-well plate loaded with 1 µl of cDNA and a final concentration of 0.5 µM of each forward and reverse primers in a final volume of 10 µl. Each PCR run included also a four-points serial dilution curve and non-retrotranscribed-RNA and water as negative controls. The RT-qPCR cycling conditions consisted of an initial denaturation at 95 °C for 30 s and 40 cycles of a two-step amplification program (95 °C for 5 s and 60 °C for 30 s). A melting curve was systematically monitored (temperature gradient at 0.5 °C/5 s from 70 to 90 °C) at the end of each run to confirm the specificity of the amplification reaction. The gene expression of *npy*, *pomc*, *hcrt*, *cartpt-I*, and *cartpt-II*, was quantified in the hypothalamus. In the liver, we analyzed the gene expression of *leptin aI*, the gluconeogenic enzyme *pck2a*, the glucose transporters *slc2a2* and *slc5a1*, the transcription factors *ppara*, *pparg*, *nr1d1*, and *nr1d2 b*, and the clock genes *per1a*, *per1b*, *per2a*, *per3*, *clock1a*, and *bmal1a*. The Gene Data Bank reference numbers and the primers (Sigma-Aldrich) sequences employed for all target and reference genes elongation factor 1α (*eef1a1*), β-actin (*actb*), and ribosomal 18S (*r18S*) are shown in Table 1. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression (fold change). Data obtained were normalized to the vehicle group in each experiment.

Table 1. Primer sequences used in the RT-qPCR assays. Accession numbers and primer sequences of the genes employed in the RT-qPCR assays.

Gene	Accession number		Primer sequence 5′ → 3′	Product (bp)
npy	M87297	Forward	TTCGTCTGCTTGGGAACTCT	151
		Reverse	TGGACCTTTTGCCATACCTC	
pomc	AJ431209	Forward	CTCACCACTGACGAGAACATCTTG	121
		Reverse	CGGTTTGCTCCAGCTCAGA	
cartpt-1	AY033816	Forward	GTGCCGAGATGGACTTTGAC	97
		Reverse	AGCTGCTTCTCGTTGGTCAG	
cartpt-II	AY033817	Forward	GGAAAAGCTGCAGACGAAAC	107
		Reverse	CGATTCGAGAGCCTTTTCTG	
hcrt	DQ923590	Forward	ACTGCACAGCCAAGAGAGTTCA	188
		Reverse	GTTATTAAAGCGGCCGATATGC	
lep al	FJ534535	Forward	AGCTCCTCATAGGGGATC	192
		Reverse	TAGATGTCGTTCTTTCCTTA	
pck2a	MK598557	Forward	TAACTGGCGTCATGGTGTGT	120
		Reverse	TAGCCGAAGAAAGGACGCAT	
slc2a2	DQ098687	Forward	TGTGCTGTGGCCATGAC	113
		Reverse	CCAGGTCCGATCTCAAAGAA	
slc5a1	JN867793	Forward	GATCGTGACCATGCCAGAG	156
		Reverse	TTTAGTCCCAGAGCCTGGTT	
ppara	AY198322	Forward	CCATCCCGACAACGAGTTCC	121
		Reverse	CAGCGACGTGTCTTCTGTCT	
pparg	AY894893	Forward	TTCCACAGCTGTCAGTCTCG	201
		Reverse	CCTACGGACAGATCTTCATG	
nr1d1	KU242427	Forward	CGTTCATCTCAGGCACCACT	166
		Reverse	AACTGACCTGCAGACACCAG	
nr1d2 b	MH674345	Forward	AGCTGCAAGCTCTGAACCTC	164
		Reverse	GTTGGGGTGGTTCTTGGTGA	
per1a	EF690698	Forward	CAGTGGCTCGAATGAGCACCA	155
		Reverse	TGAAGACCTGCTGTCCGTTGG	
per1b	KP663726	Forward	CTCGCAGCTCCACAAACCTA	235
		Reverse	TGATCGTGCAGAAGGAGCCG	
per2a	EF690697	Forward	TTTGTCAATCCCTGGAGCCGC	116
		Reverse	AAGGATTTGCCCTCAGCCACG	
per3	EF690699	Forward	GGCTATGGCAGTCTGGCTAGTAA	130
		Reverse	CAGCACAAAACCGCTGCAATGTC	
clock1a	KJ574204	Forward	CGATGGCAGCATCTCTTGTGT	187
		Reverse	TCCTGGATCTGCCGCAGTTCAT	
bmal1a	KF840401	Forward	AGATTCTGTTCGTCTCGGAG	161
		Reverse	ATCGATGAGTCGTTCCCGTG	
eef1a1	AB056104	Forward	CCCTGGCCACAGAGATTTCA	101
		Reverse	CAGCCTCGAACTCACCAACA	
actb	AB039726	Forward	CAGGGAGTGATGGTTGGCA	168
		Reverse	AACACGCAGCTCGTTGTAGA	
r18S	FJ710820	Forward	ATGATTAAGAGGGACGGCCGG	146
		Reverse	TGATCGTCTTCGAACCTCCGA	

npy, neuropeptide Y; *pomc*, pro-opio melanocortin; *cartpt*, cocaine- and amphetamine-regulated transcript; *hcrt*, pre-proorexin; *lep al*, leptin aI; *pck2a*, phosphoenolpyruvate carboxykinase 2a; *slc2a2*, glucose facilitated transporter type 2 (GLUT2); *slc5a1*, sodium-glucose linked transporter 1; *ppara* and *b*, peroxisome proliferator-activated receptor type α and type γ; *nr1d1*, rev-erbα; *nr1d2 b*, rev-erbβ b; *per*, period; *bmal1a*, brain and muscle ARNT-like 1a; *clock1a*, circadian locomotor output cycles kaput 1a; *eef1a1*, elongation factor 1α; *actb*, β-actin; and *r18S*, ribosomal 18S.

2.5. Statistics

Data are presented as mean ± S.E.M. The existence of significant periods in daily locomotor activity was analyzed by constructing chi-square periodograms with a significance level set at 0.05 (EL TEMPS®, Prof. Antoni Díez Noguera, University of Barcelona). One-way analysis of variance (ANOVA) for repeated measures was used for the analysis of body weight gain. The daily effects of PEA on body weight was analyzed by one-way ANOVA. For the nutritional indexes a two-way ANOVA was performed with experimental time (first and last day) and treatment (Control and PEA) as main factors. One-way ANOVA was performed to compare the remaining parameters here analyzed (Control and PEA). Saphiro-Wilk and Levene tests were used to confirm normality and homocedasticity of the data, respectively. When necessary, data were transformed to logarithmic or square root scale to fulfil the conditions of normality and homocedasticity. In case of a significant effect ($p < 0.05$), post hoc comparisons using Student-Newman-Keuls (SNK) test were employed. Statistical analyses were performed by SigmaPlot 12.0 statistics package and IBM SPSS 22.0 software package (SPSS Inc., Chicago, IL, USA).

3. Results

The effects of PEA on food intake and body weight gain in goldfish are presented in **Fig. 1**. Acute PEA treatment induced a significant decrease in food intake at 6 h ($p = 0.017$) and 8 h ($p = 0.003$) post-injection (**Fig. 1A**). These reductions were approx. 37 % at 6 h and 50 % at 8 h after PEA treatment. Body weight was significantly reduced in goldfish injected with PEA for 10 days (**Fig. 1B**). PEA significantly impaired body weight gain [$F(1, 22) = 79.439$; $p < 0.001$], and this effect was observed from second day of treatment onwards. Specific growth rate was significantly lower ($p < 0.001$) after chronic PEA treatment ($SGR_{CONTROL}$ = 1.69 ± 0.14 %/day; SGR_{PEA} = - 0.31 ± 0.14 %/day) The PEA treatment for 10 days significantly impaired the increase in nutritional index observed in control fish (initial $NI_{CONTROL}$ = 3.05 ± 0.08 % *vs.* final $NI_{CONTROL}$ = 3.37 ± 0.10 %, p = 0.023; initial NI_{PEA} = 3.28 ± 0.11 % *vs.* final NI_{PEA} = 3.21 ± 0.10 %, p = 0.617).

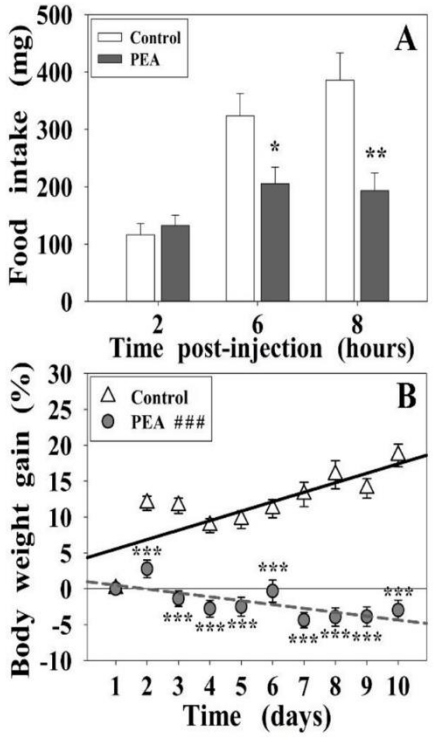


Fig. 1. Food intake and body weight in goldish after PEA treatment. (**A**) Food intake at 2, 6 and 8 h after acute i.p. administration of vehicle alone (Control) or containing PEA (20 μg.g^{-1} bw). (**B**) Body weight gain in fish daily i.p. injected with vehicle alone (Control) or containing PEA (20 μg/g bw) for 10 days. Data are expressed as mean ± S.E.M. (n = 10-12 fish per group). Continuous black and dashed grey lines in Figure B indicate the linear regression of data from Control and PEA groups, respectively. ### $p < 0.001$ between Control and PEA groups (one-way ANOVA for repeated measures); ** $p < 0.01$, and *** $p < 0.001$ PEA-treated compared to Control group in the same day (one-way ANOVA, *post hoc* SNK).

Locomotor activity recordings in control and PEA-treated goldfish for 10-days are shown in **Fig. 2**. Daily average waveforms showed that general- and feeding-related locomotor activities were entrained to the 12L:12D cycle in both groups of fish. A diurnal rhythmic pattern with high locomotor activity during daytime and low activity during nighttime was found (**Figs. 2A**, **B**, **D**, and **E**). In fact, circadian periods (τ) of both, general and feeding-related locomotor activity, were identical (τ = 24.2 h) in control and PEA-treated fish. A peak in activity is observed prior food delivery (10 a.m.), considered as FAA (**Figs. 2A**, **B**, and **D**), except in the **Fig. 2E**, which represent the feeding-related locomotor activity of PEA-treated

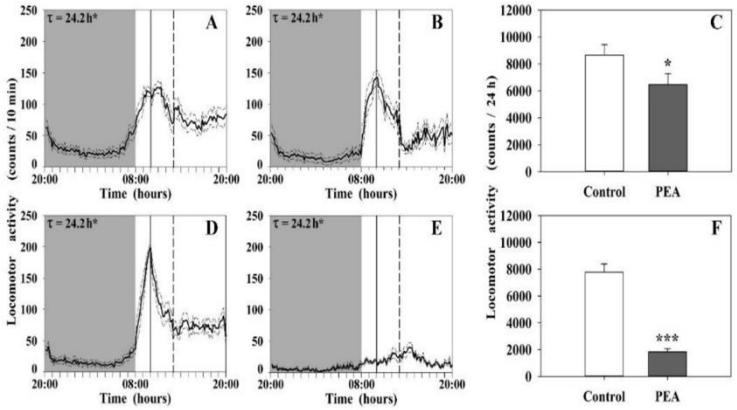


Fig. 2. Locomotor activity of goldfish after chronic PEA treatment. Representative average waveforms express the mean ± S.E.M. of the activity recorded through 24 h periods over the 10 days of experiment of the general locomotor activity (**A**, Control; **B**, PEA), and feeding-related locomotor activity (**D**, Control; **E**, PEA) after i.p. administration of vehicle alone (Control) or containing PEA (20 μg.g^{-1} bw). Histograms represent the mean + S.E.M. of the total amount of the activity recorded during each day of general (**C**) and feeding- (**F**) related locomotor activity in both experimental groups (Control and PEA). Grey area corresponds to the night period, continuous line indicates the exact time of feeding, dashed line indicates the exact time of the injections, and τ indicates the period of the rhythms when significant (*). In **C** and **F**, * $p < 0.05$ and *** $p < 0.001$ PEA-treated compared to Control group (one-way ANOVA, *post hoc* SNK).

fish. In this figure, FAA measured by two photocells located below the automatic feeder was significantly lower ($p < 0.001$) after chronic PEA treatment ($FAA_{CONTROL}$ = 2385 ± 111 counts/3 h; FAA_{PEA} = 288 ± 25 counts/3 h), which did not exhibit this well-known activity increase entrained by feeding time. The PEA treatment (10 days) induced a significant reduction (25 %) in the daily mean of general locomotor activity ($p = 0.027$), and a drastic decrease (76 %) in feeding-related locomotor activity ($p < 0.001$) compared to activity of control fish (**Figs. 2C** and **F**, respectively).

The effects of acute PEA administration on hypothalamic and hepatic mRNA abundance of some feeding regulators are summarized in **Fig. 3**. The PEA treatment significantly decreased mRNA abundance of hypothalamic *npy* ($p = 0.010$; **Fig. 3A**) and increased that of hepatic *lep al* ($p < 0.001$; **Fig. 3F**), while the abundance of the other transcripts (*pomc*, *hcrt*, *cartpt-I*, and *cartpt-II*) did not show significant changes.

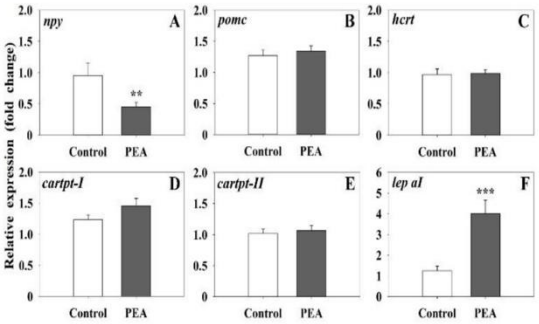


Fig. 3. Effects of PEA on mRNA abundance of feeding regulators in goldfish. Relative mRNA abundance of hypothalamic *npy* (**A**), *pomc* (**B**), *hcrt* (**C**), *cartpt-I* (**D**), *cartpt-II* (**E**), and hepatic *lep al* (**F**) after i.p. administration of vehicle alone (Control) or containing PEA (20 μg.g^{-1} bw) after 6 h post-injection. Data are expressed as mean + S.E.M. (n = 8-10/group). ** $p < 0.01$ and *** $p < 0.001$ PEA-treated compared to Control group (one-way ANOVA, *post hoc* SNK).

Plasma levels and hepatic concentration of metabolites are shown in **Fig. 4**. No significant differences occurred in plasma levels of fatty acid, triglyceride, and glucose after acute and chronic PEA administration (**Figs. 4A-C**). The concentration of hepatic triglyceride (**Fig. 4E**) and lactate (**Fig. 4G**) decreased 6-h post-injection in PEA-treated compared to control fish (p = 0.003, for both metabolites). The i.p. treatment with PEA did not modify hepatic concentration of fatty acid, total lipid, glucose, and glycogen in the acute experiment. The PEA chronic treatment did not alter concentrations of any metabolite in liver.

Fig. 5 summarizes the effects of PEA on the activity of liver enzymes involved in lipid metabolism. The activity of FAS (**Fig. 5B**) increased after chronic PEA treatment (p = 0.016), but no changes occurred in the acute experiment. No significant changes were found for ACLY, CPT-1, and HOAD activities in the liver after PEA administration in both acute and chronic experiments.

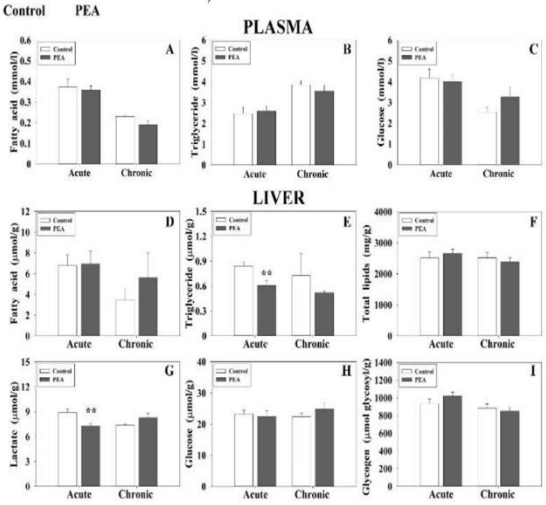


Fig. 4. Effects of PEA on levels of plasmatic and hepatic metabolites in goldfish. Fatty acid (**A**), triglyceride (**B**), and glucose (**C**) in plasma; and fatty acid (**D**), triglyceride (**E**), total lipid (**F**), lactate (**G**), glucose (**H**), and glycogen (**I**) in liver after i.p. administration of vehicle alone (Control) or containing PEA (20 μg.g^{-1} bw) in acute (6 h post-injection) and chronic (10 days) experiments. Data are expressed as mean + S.E.M. (n = 8-10/group). ** $p < 0.01$ PEA-treated compared to Control group (one-way ANOVA, *post hoc* SNK).

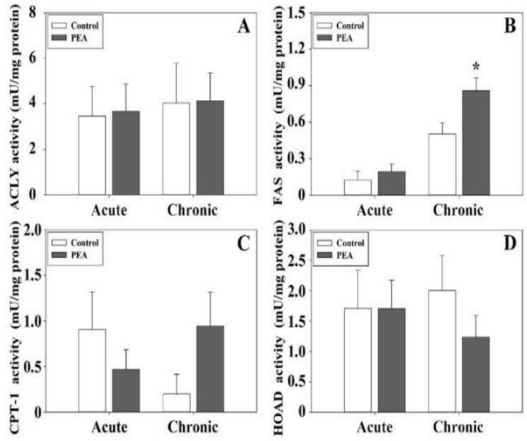


Fig. 5. Effects of PEA on activity of enzymes involved in lipid metabolism in goldfish liver. ACLY (**A**), FAS (**B**), CPT-1 (**C**), and HOAD (**D**) activities after i.p. administration of vehicle alone (Control) or containing PEA (20 μg.g^{-1} bw) in acute (6 h post-injection) and chronic (10 days) experiments. Data are expressed as mean + S.E.M. (n = 8-10/group). * $p < 0.05$ PEA-treated compared to Control group (one-way ANOVA, *post hoc* SNK).

The activity of enzymes involved in glucose metabolism assessed in the liver after acute and chronic PEA administration is shown in

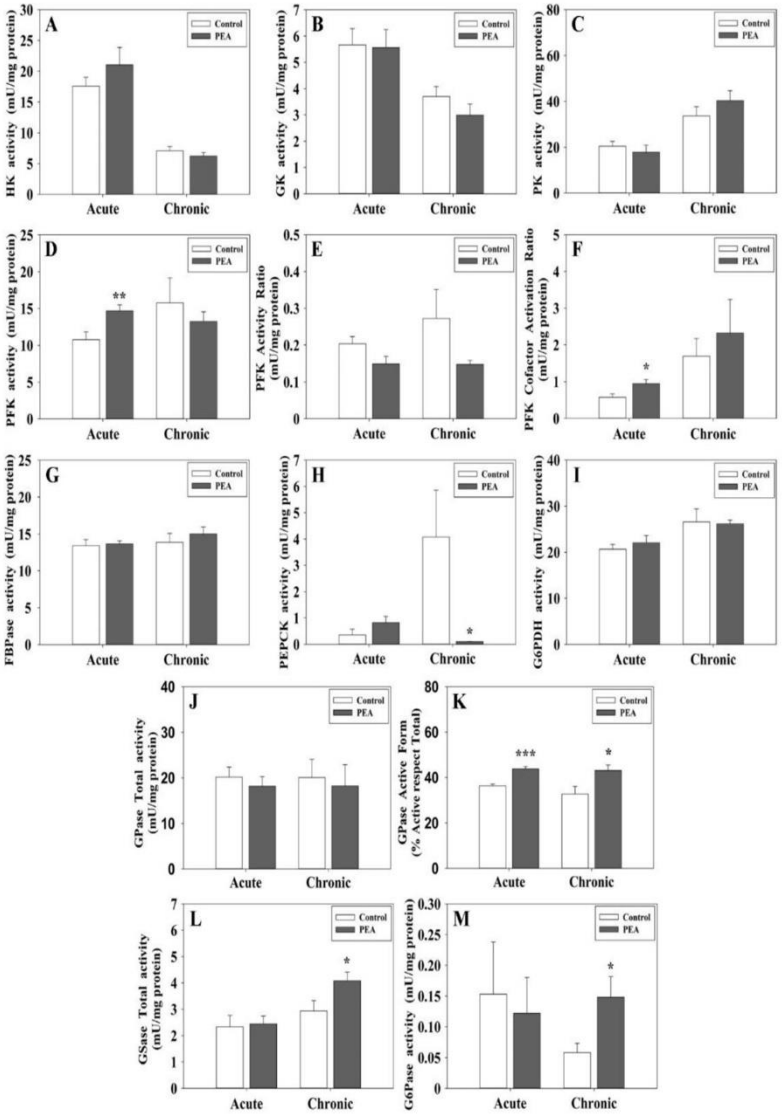


Fig. 6. Effects of PEA on activity of enzymes involved in glucose metabolism in goldfish liver. HK (**A**), GK (**B**), PK (**C**), PFK (**D-F**), FBPase (**G**), PEPCK (**H**), G6PDH (**I**), GPase (**J-K**), GSase (**L**), and G6Pase (**M**) activities after i.p. administration of vehicle alone (Control) or containing PEA (20 µg.g^{-1} bw) in acute (6 h post-injection) and chronic (10 days) experiments. Data are expressed as mean + S.E.M. (n = 8-10/group). ** $p < 0.01$ PEA-treated compared to Control group (one-way ANOVA, *post hoc* SNK).

Fig. 6. The activity of both PFK and its cofactor activation ratio (**Figs. 6D** and **F**, respectively) was higher in PEA than in control group in the acute experiment (p = 0.006 and p = 0.015, respectively). PEPCK activity (**Fig. 6H**) decreased after chronic PEA treatment (p = 0.013). The percentage of GPase in the active form (**Fig. 6K**) increased after PEA treatment in both experiments ($p < 0.001$ for acute and p = 0.013 for chronic). The total activity of GSase (**Fig. 6L**) and the G6Pase (**Fig. 6M**) increased after chronic PEA treatment (p = 0.033 and p = 0.042, respectively). No significant differences due to PEA treatment were observed in the remaining enzymatic activities assessed.

The effects of peripheral PEA treatment on mRNA abundance of glucose metabolism-related genes are shown in **Fig. 7**. Chronic treatment with PEA increased the expression of *slc2a2* (p = 0.041; **Fig. 7B**), while acute PEA treatment increased the expression of *slc5a1* (p = 0.027; **Fig. 7C**). No significant differences were noted for *pck2a* relative mRNA expression (**Fig. 7A**).

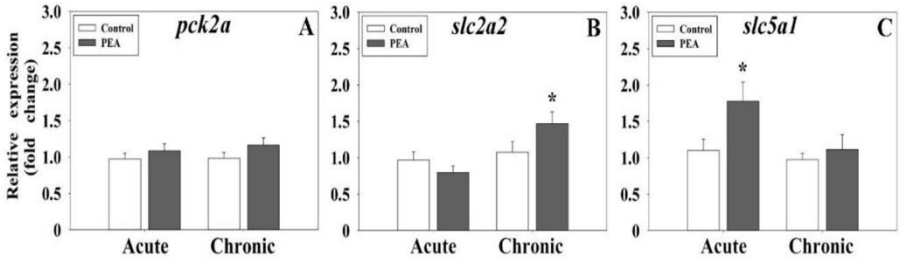


Fig. 7. Effects of PEA on mRNA abundance of genes related to hepatic metabolism. Relative mRNA abundance of *pck2a* (**A**), *slc2a2* (**B**), and *sl5a1* (**C**) after i.p. administration of vehicle alone (Control) or containing PEA (20 µg.g^{-1} bw) in acute (6 h post-injection) and chronic (10 days) experiments. Data obtained by RT-qPCR are shown as mean + S.E.M. (n = 8-10/group) in relative units ($2^{-\Delta\Delta Ct}$ method; PEA group is relativized to respective Control group). * $p < 0.05$ PEA-treated compared to Control group (one-way ANOVA, *post hoc* SNK).

The effects of peripheral PEA treatment on hepatic expression of transcription factors are shown in **Fig. 8**. Both acute and chronic PEA treatments significantly increased the relative mRNA expression of *nr1d1* (p = 0.028 and p = 0.003, for acute and chronic treatment, respectively; **Fig. 8C**), whereas the remaining transcripts (*ppara*, *pparg*, and *nr1d2 b*) did not show significant changes in abundance after PEA treatment.

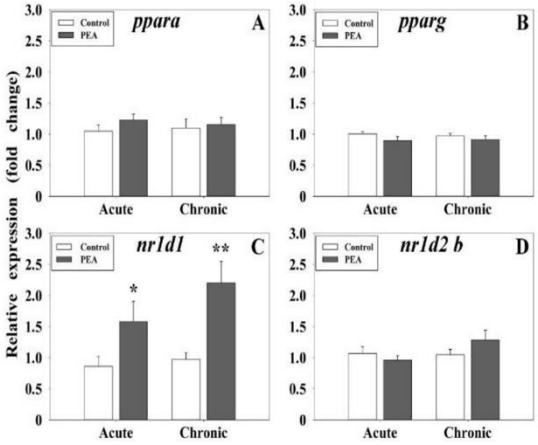


Fig. 8. Effects of PEA on mRNA abundance of hepatic transcription factors in goldfish**.** Relative mRNA abundance of *ppara* (**A**), *pparg* (**B**), *nr1d1* (**C**), and *nr1d2 b* (**D**) after i.p. administration of vehicle alone (Control) or containing PEA (20 μg.g^{-1} bw) in acute (6 h post-injection) and chronic (10 days) experiments. Data obtained by RT-qPCR are shown as mean + S.E.M. (n = 8-10/group) in relative units ($2^{-\Delta\Delta Ct}$ method; PEA group is relativized to respective Control group). * $p < 0.05$ and ** $p < 0.01$ PEA-treated compared to Control group (one-way ANOVA, *post hoc* SNK).

Fig. 9 shows hepatic mRNA abundance of clock genes after PEA administration. The values of *per1a* (**Fig. 9A**) increased after both acute (p = 0.002) and chronic (p = 0.003) PEA treatments. Values of *per1b* and *per2a* did not show any significant difference (**Figs. 9B** and **C**). Acute PEA treatment caused a decrease in the values of *per3* (p = 0.012; **Fig. 9D**), but it did not the chronic treatment. The chronic administration of PEA induced an increase in values of *clock1a* (p < 0.001) and *bmal1a* (p = 0.001) (**Figs. 9E** and **F**, respectively), without significant modifications by the acute treatment.

4. Discussion

PEA inhibits energy inflow through decreased food intake

PEA administration resulted in a clear decrease in food intake of goldfish 6 and 8 h after treatment. This anorectic effect of PEA is comparable to that demonstrated in mammals (Mattace Raso et al., 2014b; Rodríguez de Fonseca et al., 2001), as well as to that occurring in goldfish after OEA treatment (Tinoco et al., 2014). The anorectic effect of NAEs fits well with their role as satiety signals in vertebrates, including fish, according to their postprandial increase produced in the gastrointestinal tissues (Astarita et al., 2006; Gómez-Boronat et al., 2019; Petersen et al., 2006), so limiting food intake is a logical effect for them.

Homeostatic changes in food intake in fish are the response of hypothalamic integration of metabolic and endocrine signals resulting in changes in the expression of orexigenic and anorexigenic neuropeptides (Delgado et al., 2017; Soengas et al., 2018). Thus, the anorectic actions of PEA can be mediated through the modulation of central and/or peripheral signals involved in the feeding regulation. In the present study, we have assessed changes in hypothalamic mRNA abundance of several neuropeptides including the orexigenic *npy* and *hcrt*, and the anorectics *pomc*, *cartpt-I*, and *cartpt-II*. The clear reduction occurred in the expression of the orexigenic *npy* after PEA treatment suggests that the anorectic response elicited by PEA might be mediated by the decrease of this neuropeptide at hypothalamic level. Decreased mRNA abundance of *npy* has been observed in hypothalamus of several fish species under conditions eliciting anorectic responses, such as the effects of raised levels of nutrients or anorectic hormones (Delgado et al., 2017; Soengas et al., 2018). However, it is interesting that other anorectic responses in fish displayed in hypothalamus at the same time decreased levels of orexigenics and increased levels of anorectics (Delgado et al., 2017; Soengas et al., 2018), although in this study no changes occurred in mRNA abundance of anorectics *pomc* and *cartpt*. This clear differential effect of PEA treatment compared with other anorectic conditions could be related to the involvement of NAEs, including PEA, in food intake regulation through hedonic rather than homeostatic mechanisms, as suggested Monteleone et al. (2016). The absence of changes in mRNA abundance of hypothalamic anorectics neuropeptides is different than that known in rat where increased levels of *pomc* occurred after PEA treatment (Mattace Raso et al., 2014b). This apparent discrepancy between our results in fish and previous in mammals might result from species-specific differences, treatment duration (10 days in fish *vs.* 5 weeks in rats) or differences in experimental approaches (wild-type fish vs. ovariectomy-induced model of obesity in rats). At peripheral level, PEA induced a sharp increase in mRNA abundance of *lep al* in goldfish liver, allowing us to suggest a rise in circulating levels of leptin after PEA treatment. PEA is known to increase hypothalamic leptin signaling through STAT3 phosphorylation in rats (Mattace Raso et al., 2014a), although PEA did not modify plasma leptin in sham-operated rats or reverted the increased leptin levels in ovariectomy obese rats (Mattace Raso et al., 2014b). Thus, the hypothetical raised levels of leptin once integrated in hypothalamus could be responsible of the sharp decrease observed in the hypothalamic *npy* mRNA abundance. Accordingly, leptin treatment is known to induce a decrease in mRNA abundance of *npy*

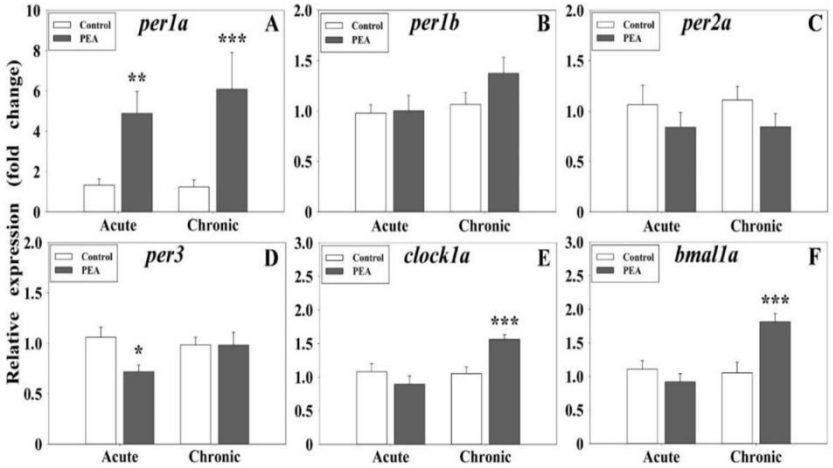


Fig. 9. Effects of PEA on gene expression of hepatic clock genes in goldfish. Relative expression of *per1a* (**A**), *per1b* (**B**), *per2a* (**C**), *per3* (**D**), *clock1a* (**E**), and *bmal1a* (**F**) after i.p. administration of vehicle alone (Control) or containing PEA (20 μg.g^{-1} bw) in acute (6 h post-injection) and chronic (10 days) experiments. Data obtained by RT-qPCR are shown as mean + S.E.M. (n = 8-10/group) in relative units ($2^{-\Delta\Delta Ct}$ method; PEA group is relativized to respective Control group). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ PEA-treated compared to Control group (one-way ANOVA, *post hoc* SNK).

in fish hypothalamus, as demonstrated in goldfish (Volkoff et al., 2003) and rainbow trout (Aguilar et al., 2011; Murashita et al., 2008). The presence in fish of leptin receptors in hypothalamus (Angotzi et al., 2016; Tinoco et al., 2012), as well as PEA receptors such as PPARα in liver (Gómez-Boronat et al., 2019, 2016; Librán-Pérez et al., 2015a, 2015b) give further support to this hypothesis.

On the other hand, the decrease in food intake would be responsible of the reduction in body weight, growth rate, and nutritional index observed in fish chronically-treated with PEA for 10 days. A similar decrease in these parameters was also observed after treatment with leptin for 10 days in the same species, fitting with the possible increase in leptin induced by PEA above discussed. This negative weight gain induced by PEA in goldfish is comparable to that observed in mammals. Thus, a decrease in body weight was observed after PEA treatment in obese rats (Mattace Raso et al., 2014b), whereas high levels of NAEs including PEA in the milk of humans provoked a lower weight gain in four-months-old infants (Bruun et al., 2018). These effects seems to be extended to other NAEs since chronic treatment with OEA also resulted in a decrease in weight in goldfish (Gómez-Boronat et al., 2016) in a way comparable to that of mammals (Hansen and Diep, 2009). These results reinforce the role of PEA and OEA as signals of nutritional status that can regulate feeding and body mass homeostasis in vertebrates.

PEA can modify energy expenditure through changes in locomotor activity

Fish treated with PEA displayed a clear decrease in locomotor activity, either general or related to feeding. This decrease in the general locomotor activity has been observed before in mice (Zambrana-Infantes et al., 2018) and it is comparable to the decrease found after OEA treatment in fish (Gómez-Boronat et al., 2016; Tinoco et al., 2014) and mammals (Rodríguez de Fonseca et al., 2001; Proulx et al., 2005). The decrease related to feeding could be a logical consequence of the decrease observed in food intake as discussed above, but this would mainly justify the reduction in locomotion after mealtime (approximately between 10 a.m.-13 p.m.), but not before (FAA, 7-10 a.m.). The inhibition of FAA found in the present study after PEA treatment suggests that this NAE can be acting by decreasing first phases of eating behavior, such as arousal and/or appetitive phases, as previously described to other anorectic as melatonin in goldfish (Azpeleta et al., 2010). Thus, PEA would act both in the early and in the consummatory phases of feeding behavior in fish. On the other hand, alterations in the amount of FAA following drug treatment could be mediated by modifications in the clock mechanism. In this sense, PEA has modified the hepatic expression of some clock genes (*per1a*, *clock1a*, *bmal1a*, and *nr1d1* increased; and *per3* reduced) in the present study. Nevertheless, to confirm the hypothesis that PEA affects circadian clock, future studies are required to demonstrate that a property of the clock, such as its phase or period, has been affected after drug treatment. In summary, it can be suggested that PEA may play a role in regulating locomotor activity and therefore energy expenditure in fish, as reported in mammals.

Hepatic metabolism is altered by PEA treatment

Another important aspect related to energy expenditure regards changes in energy metabolism especially in tissues like liver. There is no direct evidence in any vertebrate species concerning the impact of PEA on liver glucose metabolism. However, such a relationship is likely considering that PEA levels decrease following a meal under normoglycemic conditions, but are permanently increased under hyperglycemic conditions in mammals (Matias et al., 2007). In the present study, the potential of glucose transport in liver is affected by PEA in the way that increased mRNA abundance of the two main glucose carriers, i.e. *slc2a2* and *slc5a1*, occurred after PEA treatment with differences in the activated transcript depending on the acute or chronic treatment. This increased potential of fish liver to transport glucose is however not matched by increased capacity of glucose phosphorylation, as demonstrated the absence of changes observed in the activity of HK and GK. Nevertheless, glycolytic potential was apparently enhanced after PEA treatment as evidenced the activation of PFK activity. A rise in glycolytic potential together with the increased potential of glucose to be transported into liver would suggest an enhanced use of glucose into the liver after PEA treatment. The enhanced glycolytic potential without significant changes in HK and GK activities might suggest an additional source of glucose 6-phosphate to be used through glycolysis instead of the phosphorylation through those enzymes. In this way, the increased potential of glycogenolysis, indicated by raised activity of GPase, is suggesting an enhancement in the availability of glucose 6-phosphate from glycogen stores after PEA treatment. However, the finding that glycogen levels did not display significant changes after PEA treatment suggest a rapid turnover. If glucose is increasingly used within the liver, it makes no sense that the pathways involved in glucose synthesis becomes activated. In this way, it is reasonable that a decrease occurred in the gluconeogenic potential in liver after PEA treatment as demonstrated by decreased PEPCK activity. Finally, G6Pase activity increased after chronic (but not acute) PEA treatment allowing us to suggest that glucose export capacity in liver can be altered by PEA treatment. Considering that GLUT2 is a reversible carrier (i.e. either introducing glucose into the liver or releasing it from liver), the increased levels of its transcript (*slc2a2*) under chronic PEA treatment would match with a hypothetical increased export capacity. As a whole, PEA treatment enhances internal glucose use in liver and part of glucose may be exported to be used in other tissues. In fact, a non-significant increase occurred in plasmatic glucose levels of fish chronically treated with PEA compared with controls. These effects are comparable to those elicited by OEA in liver of the same species regarding gluconeogenic and glycogenolytic potentials, but not regarding glucose phosphorylation and use (Gómez-Boronat et al., 2016), clearly suggesting differential regulatory effects for both NAEs in glucose metabolism of goldfish liver.

In lipid metabolism, the only relevant change was the increased activity of FAS after chronic PEA treatment suggesting enhanced lipogenic potential. Considering that enhanced glucose use occurred simultaneously, we may suggest that at least part of this glucose might be being used for lipid biosynthesis (Rui, 2014). A comparable rise in the lipogenic potential of liver was also observed in the same species after acute, but not chronic treatment with OEA (Gómez-Boronat et al., 2016). The few available studies carried out in adipose tissue of mammals clearly suggested that lipid metabolism was affected by PEA treatment, especially regarding lipolysis and β-oxidation (Mattace Raso et al., 2014b). These differences in NAEs effects on lipid metabolism between fish and mammals can be due to species-specific differences, different analyzed tissue (liver vs. adipose tissue) or different methodological approaches (enzymatic activity vs. enzyme expression). Lipogenic action induced by PEA in the present study might relate to the increase observed in mRNA abundance of *bmal1a*, a clock gene that, at least in mammals, is also involved in the stimulation of lipogenesis in liver (Zhang et al., 2014). Similarly, OEA also elicited parallel changes in the mRNA abundance of liver bmal1a and the activation of the lipogenic pathway (Gómez-Boronat et al., 2016). Further support for this relationship comes from studies carried out in the liver of another fish species such as rainbow trout describing that changes in the mRNA abundance of lipogenic enzymes (Hernández-Pérez et al., 2015) and clock genes like *bmal1* (Hernández-Pérez et al., 2017) are in phase, suggesting a relationship between them.

Multiple mechanisms can be involved in PEA actions in the liver. PEA, as other NAEs, can activate several different receptors, including PPARα, transient receptor potential vanilloid type 1 (TRPV1), and G protein-coupled receptor GPR119 (Hansen, 2010; Kleberg et al., 2014), that may be involved in modulating hepatic metabolism. Taking in mind that PPARα directly regulates the transcription of BMAL1 and REV-ERBα in mammals (Charoensuksai and Xu, 2010; Chen and Yang, 2014), it is possible that the increase in the hepatic expression of these clock genes (*bmal1a* and *nr1d1*) after PEA treatment in goldfish can be mediated via PPARα activation. It cannot be discarded that PEA action on hepatic metabolism can be mediated by interactions with hormones involved in metabolism regulation, e.g. modifications in hepatic leptin in the present study.

In summary, we have assessed for the first time in fish the impact of PEA on several components of energy homeostasis. On the side of energy inflow, we demonstrated that PEA treatment induced a decrease in food intake, probably mediated by leptin increase and NPY decrease. The reduced food intake is also reflected in a reduction in weight gain, growth rate, and nutritional index in PEA-treated fish. On the side of energy outflow, we observed a marked reduction of locomotor activity either in general or in that related to feeding. At the level of metabolism, we observed that PEA induced changes in glucose metabolism in the liver consisting in an/the increased use of glucose not only to be used in the tissue, but also probably to be exported and used in other tissues. The lipogenic capacity in the liver increased after PEA treatment, a different result to that reported in mammals, but similar to that described with OEA in the same species. As a whole, the present study displayed a global picture of the action of PEA in several compartments related to energy homeostasis supporting a regulatory role for this NAE different in many aspects to that already known in mammals.

Acknowledgements

This study was supported by grants from Spanish Agencia Estatal de Investigación (AEI) to M.J.D. (AGL2016-74857-C3-2-R) and by AEI and European Fund of Regional Development (FEDER) to JLS (AGL2016-74857-C3-1-R). MG-B was recipient of a predoctoral fellow from AEI (BES-2014-068103). M.C-S was recipient of a postdoctoral fellowship from Xunta de Galicia (ED481B2018/018). The authors belong to the Fish Welfare and Stress Network from AEI (AGL2016-81808-REDT).

References

Adamovich, Y., Aviram, R., Asher, G., 2015. The emerging roles of lipids in circadian control. Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids 1851, 1017–1025. https://doi.org/10.1016/j.bbalip.2014.11.013

Aguilar, A.J., Conde-Sieira, M., López-Patiño, M.A., Míguez, J.M., Soengas, J.L., 2011. *In vitro* leptin treatment of rainbow trout hypothalamus and hindbrain affects glucosensing and gene expression of neuropeptides involved in food intake regulation. Peptides 32, 232–240. https://doi.org/10.1016/j.peptides.2010.11.007

Albrecht, U., Ripperger, J.A., 2018. Circadian clocks and sleep: Impact of rhythmic metabolism and waste clearance on the brain. Trends Neurosci. 41, 677–688. https://doi.org/10.1016/j.tins.2018.07.007

Angotzi, A.R., Stefansson, S.O., Nilsen, T.O., Øvrebø, Jan.I., Andersson, E., Taranger, G.L., Rønnestad, I., 2016. Identification of a novel leptin receptor duplicate in Atlantic salmon: Expression analyses in different life stages and in response to feeding status. Gen. Comp. Endocrinol. 235, 108–119. https://doi.org/10.1016/j.ygcen.2016.06.004

Astarita, G., Rourke, B.C., Andersen, J.B., Fu, J., Kim, J.H., Bennett, A.F., Hicks, J.W., Piomelli, D., 2006. Postprandial increase of oleoylethanolamide mobilization in small intestine of the Burmese python (*Python molurus*). Am. J. Physiol.-Regul. Integr. Comp. Physiol. 290, R1407–R1412. https://doi.org/10.1152/ajpregu.00664.2005

Azpeleta, C., Martínez-Álvarez, R.M., Delgado, M.J., Isorna, E., De Pedro, N., 2010. Melatonin reduces locomotor activity and circulating cortisol in goldfish. Horm. Behav. 57, 323–329. https://doi.org/10.1016/j.yhbeh.2010.01.001

Bruun, S., Gouveia-Figueira, S., Domellöf, M., Husby, S., Neergaard Jacobsen, L., Michaelsen, K.F., Fowler, C.J., Zachariassen, G., 2018. Satiety factors oleoylethanolamide, stearoylethanolamide, and palmitoylethanolamide in mother's milk are strongly associated with infant weight at four months of age—Data from the Odense child cohort. Nutrients 10, 1747. https://doi.org/10.3390/nu10111747

Charoensuksai, P., Xu, W., 2010. PPARs in rhythmic metabolic regulation and implications in health and disease. PPAR Res. 2010. https://doi.org/10.1155/2010/243643

Chen, L., Yang, G., 2014. PPARs integrate the mammalian clock and energy metabolism. PPAR Res. 2014. https://doi.org/10.1155/2014/653017

Chen, Z., Zhang, Y., Guo, L., Dosoky, N., Ferra, L. de, Peters, S., Niswender, K.D., Davies, S.S., 2017. Leptogenic effects of NAPE require activity of NAPE-hydrolyzing phospholipase D. J. Lipid Res. 58, 1624–1635. https://doi.org/10.1194/jlr.M076513

Cho, H., Zhao, X., Hatori, M., Yu, R.T., Barish, G.D., Lam, M.T., Chong, L.-W., DiTacchio, L., Atkins, A.R., Glass, C.K., Liddle, C., Auwerx, J., Downes, M., Panda, S., Evans, R.M., 2012. Regulation of circadian behavior and metabolism by rev-erbα and rev-erbβ. Nature 485, 123–127. https://doi.org/10.1038/nature11048

Conde-Sieira, M., Capelli, V., Álvarez-Otero, R., Comesaña, S., Liñares-Pose, L., Velasco, C., López, M., Soengas, J.L., 2018. Differential role of hypothalamic AMPKα isoforms in fish: an evolutive perspective. Mol. Neurobiol. https://doi.org/10.1007/s12035-018-1434-9

de Pedro, N. de, Martínez-Álvarez, R., Delgado, M.J., 2006. Acute and chronic leptin reduces food intake and body weight in goldfish (Carassius auratus). J. Endocrinol. 188, 513–520. https://doi.org/10.1677/joe.1.06349

Delgado, M.J., Cerdá-Reverter, J.M., Soengas, J.L., 2017. Hypothalamic integration of metabolic, endocrine, and circadian signals in fish: Involvement in the control of food intake. Front. Neurosci. 11, 354. https://doi.org/10.3389/fnins.2017.00354

Diep, T.A., Madsen, A.N., Holst, B., Kristiansen, M.M., Wellner, N., Hansen, S.H., Hansen, H.S., 2011. Dietary fat decreases intestinal levels of the anorectic lipids through a fat sensor. FASEB J. 25, 765–774. https://doi.org/10.1096/fj.10-166595

Eckel-Mahan, K., Sassone-Corsi, P., 2013. Metabolism and the circadian clock converge. Physiol. Rev. 93, 107–135. https://doi.org/10.1152/physrev.00016.2012

Fu, J., Gaetani, S., Oveisi, F., LoVerme, J., Serrano, A., Rodríguez de Fonseca, F., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G., Piomelli, D., 2003. Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. Nature 425, 90–93. https://doi.org/10.1038/nature01921

Gómez-Boronat, M., Isorna, E., Armirotti, A., Delgado, M.J., Piomelli, D., de Pedro, N., 2019. Diurnal profiles of N-acylethanolamines in goldfish brain and gastrointestinal tract: possible role of feeding. Front. Neurosci. 13, 450. https://doi.org/10.3389/fnins.2019.00450

Gómez-Boronat, M., Sáiz, N., Delgado, M.J., de Pedro, N., Isorna, E., 2018. Time-lag in feeding schedule acts as a stressor that alters circadian oscillators in goldfish. Front. Physiol. 9, 1749. https://doi.org/10.3389/fphys.2018.01749

Gómez-Boronat, M., Velasco, C., Isorna, E., de Pedro, N., Delgado, M.J., Soengas, J.L., 2016. The satiety factor oleoylethanolamide impacts hepatic lipid and glucose metabolism in goldfish. J. Comp. Physiol. B 186, 1009–1021. https://doi.org/10.1007/s00360-016-1009-x

González-Yanes, C., Serrano, A., Bermúdez-Silva, F.J., Hernández-Dominguez, M., Páez-Ochoa, M.A., Rodríguez de Fonseca, F., Sánchez-Margalet, V., 2005. Oleylethanolamide impairs glucose tolerance and inhibits insulin-stimulated glucose uptake in rat adipocytes through p38 and JNK MAPK pathways. Am. J. Physiol.-Endocrinol. Metab. 289, E923–E929. https://doi.org/10.1152/ajpendo.00555.2004

Grygiel-Górniak, B., 2014. Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications – a review. Nutr. J. 13, 17. https://doi.org/10.1186/1475-2891-13-17

Hansen, H.S., 2014. Role of anorectic N-acylethanolamines in intestinal physiology and satiety control with respect to dietary fat. Pharmacol. Res. 86, 18–25. https://doi.org/10.1016/j.phrs.2014.03.006

Hansen, H.S., 2010. Palmitoylethanolamide and other anandamide congeners. Proposed role in the diseased brain. Exp. Neurol. 224, 48–55. https://doi.org/10.1016/j.expneurol.2010.03.022

Hansen, H.S., Diep, T.A., 2009. N-acylethanolamines, anandamide and food intake. Biochem. Pharmacol. 78, 553–560. https://doi.org/10.1016/j.bcp.2009.04.024

Hansen, H.S., Vana, V., 2018. Non-endocannabinoid N-acylethanolamines and 2-monoacylglycerols in the intestine. Br. J. Pharmacol. https://doi.org/10.1111/bph.14175

Heisler, L.K., Lam, D.D., 2017. An appetite for life: brain regulation of hunger and satiety. Curr. Opin. Pharmacol. 37, 100–106. https://doi.org/10.1016/j.coph.2017.09.002

Hernández-Pérez, J., Míguez, J.M., Librán-Pérez, M., Otero-Rodiño, C., Naderi, F., Soengas, J.L., López-Patiño, M.A., 2015. Daily rhythms in activity and mRNA abundance of enzymes involved in glucose and lipid metabolism in liver of rainbow trout, *Oncorhynchus mykiss*. Influence of light and food availability. Chronobiol. Int. 32, 1391–1408. https://doi.org/10.3109/07420528.2015.1100633

Hernández-Pérez, J., Míguez, J.M., Naderi, F., Soengas, J.L., López-Patiño, M.A., 2017. Influence of light and food on the circadian clock in liver of rainbow trout, *Oncorhynchus mykiss*. Chronobiol. Int. 34, 1259–1272. https://doi.org/10.1080/07420528.2017.1361435

Keppler, D., Decker, K., 1974. Glycogen determination with amyloglucosidase, in: Methods of enzymatic analysis. Bergmeyer HU, New York, pp. 1127–1131.

Kinote, A., Faria, J.A., Roman, E.A., Solon, C., Razolli, D.S., Ignacio-Souza, L.M., Sollon, C.S., Nascimento, L.F., de Araújo, T.M., Barbosa, A.P.L., Lellis-Santos, C., Velloso, L.A., Bordin, S., Anhê, G.F., 2012. Fructose-induced hypothalamic AMPK activation stimulates hepatic PEPCK and gluconeogenesis due to increased corticosterone levels. Endocrinology 153, 3633–3645. https://doi.org/10.1210/en.2012-1341

Kleberg, K., Hassing, H.A., Hansen, H.S., 2014. Classical endocannabinoid-like compounds and their regulation by nutrients. BioFactors 40, 363–372. https://doi.org/10.1002/biof.1158

Leishman, E., Mackie, K., Luquet, S., Bradshaw, H.B., 2016. Lipidomics profile of a NAPE-PLD KO mouse provides evidence of a broader role of this enzyme in lipid metabolism in the brain. Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids 1861, 491–500. https://doi.org/10.1016/j.bbalip.2016.03.003

Librán-Pérez, M., Figueiredo-Silva, A.C., Panserat, S., Geurden, I., Míguez, J.M., Polakof, S., Soengas, J.L., 2013a. Response of hepatic lipid and glucose metabolism to a mixture or single fatty acids: Possible presence of fatty acid-sensing mechanisms. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 164, 241–248. https://doi.org/10.1016/j.cbpa.2012.09.012

Librán-Pérez, M., Geurden, I., Dias, K., Corraze, G., Panserat, S., Soengas, J.L., 2015a. Feeding rainbow trout with a lipid-enriched diet: effects on fatty acid sensing, regulation of food intake and cellular signaling pathways. J. Exp. Biol. 218, 2610–2619. https://doi.org/10.1242/jeb.123802

Librán-Pérez, M., López-Patiño, M.A., Míguez, J.M., Soengas, J.L., 2013b. *In vitro* response of putative fatty acid-sensing systems in rainbow trout liver to increased levels of oleate or octanoate. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 165, 288–294. https://doi.org/10.1016/j.cbpa.2013.03.024

Librán-Pérez, M., Otero-Rodiño, C., López-Patiño, M.A., Míguez, J.M., Soengas, J.L., 2014. Central administration of oleate or octanoate activates hypothalamic fatty acid sensing and inhibits food intake in rainbow trout. Physiol. Behav. 129, 272–279. https://doi.org/10.1016/j.physbeh.2014.02.061

Librán-Pérez, M., Polakof, S., López-Patiño, M.A., Míguez, J.M., Soengas, J.L., 2012. Evidence of a metabolic fatty acid-sensing system in the hypothalamus and Brockmann bodies of rainbow trout: implications in food intake regulation. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 302, R1340–R1350. https://doi.org/10.1152/ajpregu.00070.2012

Librán-Pérez, M., Velasco, C., Otero-Rodiño, C., López-Patiño, M.A., Míguez, M., Jesús M., Soengas, S., José L., 2015b. Effects of insulin treatment on the response to oleate and octanoate of food intake and fatty acid–sensing systems in rainbow trout. Domest. Anim. Endocrinol. 53, 124–135. https://doi.org/10.1016/j.domaniend.2015.06.004

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

López, M., Varela, L., Vázquez, M.J., Rodríguez-Cuenca, S., González, C.R., Velagapudi, V.R., Morgan, D.A., Schoenmakers, E., Agassandian, K., Lage, R., de Morentin, P.B.M., Tovar, S., Nogueiras, R., Carling, D., Lelliott, C., Gallego, R., Orešič, M., Chatterjee, K., Saha, A.K., Rahmouni, K., Diéguez, C., Vidal-Puig, A., 2010. Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance. Nat. Med. 16, 1001–1008. https://doi.org/10.1038/nm.2207

Matias, I., Gonthier, M.-P., Petrosino, S., Docimo, L., Capasso, R., Hoareau, L., Monteleone, P., Roche, R., Izzo, A.A., Di Marzo, V., 2007. Role and regulation of acylethanolamides in energy balance: focus on adipocytes and β-cells. Br. J. Pharmacol. 152, 676–690. https://doi.org/10.1038/sj.bjp.0707424

Mattace Raso, G., Russo, R., Calignano, A., Meli, R., 2014a. Palmitoylethanolamide in CNS health and disease. Pharmacol. Res. 86, 32–41. https://doi.org/10.1016/j.phrs.2014.05.006

Mattace Raso, G., Santoro, A., Russo, R., Simeoli, R., Paciello, O., Di Carlo, C., Diano, S., Calignano, A., Meli, R., 2014b. Palmitoylethanolamide prevents metabolic alterations and restores leptin sensitivity in ovariectomized rats. Endocrinology 155, 1291–1301. https://doi.org/10.1210/en.2013-1823

May-Zhang, L.S., Chen, Z., Dosoky, N.S., Yancey, P.G., Boyd, K.L., Hasty, A.H., Linton, M.F., Davies, S.S., 2019. Administration of N-acyl-phosphatidylethanolamine expressing bacteria to low density lipoprotein receptor$^{-/-}$ mice improves indices of cardiometabolic disease. Sci. Rep. 9. https://doi.org/10.1038/s41598-018-37373-1

Monteleone, A.M., Di Marzo, V., Monteleone, P., Dalle Grave, R., Aveta, T., Ghoch, M.E., Piscitelli, F., Volpe, U., Calugi, S., Maj, M., 2016. Responses of peripheral endocannabinoids and endocannabinoid-related compounds to hedonic eating in obesity. Eur. J. Nutr. 55, 1799–1805. https://doi.org/10.1007/s00394-016-1153-9

Murashita, K., Uji, S., Yamamoto, T., Rønnestad, I., Kurokawa, T., 2008. Production of recombinant leptin and its effects on food intake in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 150, 377–384. https://doi.org/10.1016/j.cbpb.2008.04.007

Otero-Rodiño, C., Velasco, C., Álvarez-Otero, R., López-Patiño, M.A., Míguez, J.M., Soengas, J.L., 2017. Changes in the levels and phosphorylation status of Akt, AMPK, CREB and FoxO1 in hypothalamus of rainbow trout under conditions of enhanced glucosensing activity. J. Exp. Biol. 220, 4410–4417. https://doi.org/10.1242/jeb.165159

Pawlak, M., Lefebvre, P., Staels, B., 2015. Molecular mechanism of PPARα action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. J. Hepatol. 62, 720–733. https://doi.org/10.1016/j.jhep.2014.10.039

Petersen, G., Sørensen, C., Schmid, P.C., Artmann, A., Tang-Christensen, M., Hansen, S.H., Larsen, P.J., Schmid, H.H.O., Hansen, H.S., 2006. Intestinal levels of anandamide and oleoylethanolamide in food-deprived rats are regulated through their precursors. Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids 1761, 143–150. https://doi.org/10.1016/j.bbalip.2005.12.011

Polakof, S., Míguez, J.M., Moon, T.W., Soengas, J.L., 2007a. Evidence for the presence of a glucosensor in hypothalamus, hindbrain, and Brockmann bodies of rainbow trout. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 292, R1657–R1666. https://doi.org/10.1152/ajpregu.00525.2006

Polakof, S., Míguez, J.M., Soengas, J.L., 2008a. Changes in food intake and glucosensing function of hypothalamus and hindbrain in rainbow trout subjected to hyperglycemic or hypoglycemic conditions. J. Comp. Physiol. A 194, 829–839. https://doi.org/10.1007/s00359-008-0354-y

Polakof, S., Míguez, J.M., Soengas, J.L., 2008b. Dietary carbohydrates induce changes in glucosensing capacity and food intake of rainbow trout. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 295, R478–R489. https://doi.org/10.1152/ajpregu.00176.2008

Polakof, S., Míguez, J.M., Soengas, J.L., 2007b. *In vitro* evidences for glucosensing capacity and mechanisms in hypothalamus, hindbrain, and Brockmann bodies of rainbow trout. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 293, R1410–R1420. https://doi.org/10.1152/ajpregu.00283.2007

Polakof, S., Panserat, S., Plagnes-Juan, E., Soengas, J.L., 2008c. Altered dietary carbohydrates significantly affect gene expression of the major glucosensing components in Brockmann bodies and hypothalamus of rainbow trout. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 295, R1077–R1088. https://doi.org/10.1152/ajpregu.90476.2008

Polakof, S., Soengas, J.L., 2008. Involvement of lactate in glucose metabolism and glucosensing function in selected tissues of rainbow trout. J. Exp. Biol. 211, 1075–1086. https://doi.org/10.1242/jeb.014050

Proulx, K., Cota, D., Castañeda, T.R., Tschöp, M.H., D'Alessio, D.A., Tso, P., Woods, S.C., Seeley, R.J., 2005. Mechanisms of oleoylethanolamide-induced changes in feeding behavior and motor activity. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 289, R729–R737. https://doi.org/10.1152/ajpregu.00029.2005

Ribas-Latre, A., Eckel-Mahan, K., 2016. Interdependence of nutrient metabolism and the circadian clock system: Importance for metabolic health. Mol. Metab. 5, 133–152. https://doi.org/10.1016/j.molmet.2015.12.006

Rodríguez de Fonseca, F., Navarro, M., Gómez, R., Escuredo, L., Nava, F., Fu, J., Murillo-Rodríguez, E., Giuffrida, A., LoVerme, J., Gaetani, S., Kathuria, S., Gall, C., Piomelli, D., 2001. An anorexic lipid mediator regulated by feeding. Nature 414, 209–212. https://doi.org/10.1038/35102582

Romano, A., Tempesta, B., Provensi, G., Passani, M.B., Gaetani, S., 2015. Central mechanisms mediating the hypophagic effects of oleoylethanolamide and N-acylphosphatidylethanolamines: different lipid signals? Front. Pharmacol. 6, 137. https://doi.org/10.3389/fphar.2015.00137

Rønnestad, I., Gomes, A.S., Murashita, K., Angotzi, R., Jönsson, E., Volkoff, H., 2017. Appetite-controlling endocrine systems in teleosts. Front. Endocrinol. 8, 73. https://doi.org/10.3389/fendo.2017.00073

Rui, L., 2014. Energy metabolism in the liver. Compr. Physiol. 4, 177–197. https://doi.org/10.1002/cphy.c130024

Rui, L., 2013. Brain regulation of energy balance and body weight. Rev. Endocr. Metab. Disord. 14, 387–407. https://doi.org/10.1007/s11154-013-9261-9

Seth, H., Axelsson, M., 2010. Sympathetic, parasympathetic and enteric regulation of the gastrointestinal vasculature in rainbow trout (*Oncorhynchus mykiss*) under normal and postprandial conditions. J. Exp. Biol. 213, 3118–3126. https://doi.org/10.1242/jeb.043612

Soengas, J.L., Cerdá-Reverter, J.M., Delgado, M.J., 2018. Central regulation of food intake in fish: an evolutionary perspective. J. Mol. Endocrinol. 60, R171–R199. https://doi.org/10.1530/JME-17-0320

Tanida, M., Yamamoto, N., Shibamoto, T., Rahmouni, K., 2013. Involvement of hypothalamic AMP-activated protein kinase in leptin-induced sympathetic nerve activation. PLoS ONE 8, e56660. https://doi.org/10.1371/journal.pone.0056660

Thabuis, C., Destaillats, F., Lambert, D.M., Muccioli, G.G., Maillot, M., Harach, T., Tissot-Favre, D., Martin, J.-C., 2011. Lipid transport function is the main target of oral oleoylethanolamide to reduce adiposity in high-fat-fed mice. J. Lipid Res. 52, 1373–1382. https://doi.org/10.1194/jlr.M013391

Tinoco, A.B., Armirotti, A., Isorna, E., Delgado, M.J., Piomelli, D., Pedro, N. de, 2014. Role of Oleoylethanolamide as a feeding regulator in goldfish. J. Exp. Biol. 217, 2761–2769. https://doi.org/10.1242/jeb.106161

Tinoco, A.B., Nisembaum, L.G., Isorna, E., Delgado, M.J., de Pedro, N., 2012. Leptins and leptin receptor expression in the goldfish (*Carassius auratus*). Regulation by food intake and fasting/overfeeding conditions. Peptides 34, 329–335. https://doi.org/10.1016/j.peptides.2012.02.001

Velasco, C., Otero-Rodiño, C., Comesaña, S., Míguez, J.M., Soengas, J.L., 2017. Hypothalamic mechanisms linking fatty acid sensing and food intake regulation in rainbow trout. J. Mol. Endocrinol. 59, 377–390. https://doi.org/10.1530/JME-17-0148

Volkoff, H., 2016. The neuroendocrine regulation of food intake in fish: A review of current knowledge. Front. Neurosci. 10, 540. https://doi.org/10.3389/fnins.2016.00540

Volkoff, H., Joy Eykelbosh, A., Ector Peter, R., 2003. Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. Brain Res. 972, 90–109. https://doi.org/10.1016/S0006-8993(03)02507-1

Zhang, D., Tong, X., Arthurs, B., Guha, A., Rui, L., Kamath, A., Inoki, K., Yin, L., 2014. Liver clock protein BMAL1 promotes *de novo* lipogenesis through insulin-mTORC2-AKT signaling. J. Biol. Chem. 289, 25925–25935. https://doi.org/10.1074/jbc.M114.567628

Zhang, Y., Fang, B., Emmett, M.J., Damle, M., Sun, Z., Feng, D., Armour, S.M., Remsberg, J.R., Jager, J., Soccio, R.E., Steger, D.J., Lazar, M.A., 2015. Discrete functions of nuclear receptor Rev-erbα couple metabolism to the clock. Science 348, 1488–1492. https://doi.org/10.1126/science.aab3021

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 07 May 2019
doi: 10.3389/fnins.2019.00450

Diurnal Profiles of *N*-Acylethanolamines in Goldfish Brain and Gastrointestinal Tract: Possible Role of Feeding

*Miguel Gómez-Boronat[1], Esther Isorna[1], Andrea Armirotti[2], María J. Delgado[1], Daniele Piomelli[3] and Nuria de Pedro[1]**

[1] Departamento de Genética, Fisiología y Microbiología, Unidad Docente de Fisiología Animal, Facultad de Biología, Universidad Complutense de Madrid, Madrid, Spain, [2] Analytical Chemistry Laboratory, Istituto Italiano di Tecnologia, Genoa, Italy, [3] Departments of Anatomy and Neurobiology, Pharmacology, and Biological Chemistry, University of California, Irvine, Irvine, CA, United States

OPEN ACCESS

Edited by:
Ishwar Parhar,
Monash University Malaysia, Malaysia

Reviewed by:
Etienne Challet,
Centre National de la Recherche Scientifique (CNRS), France
Nils Lambrecht,
University of California, Irvine, United States

***Correspondence:**
Nuria de Pedro
ndepedro@bio.ucm.es

Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience

Received: 20 December 2018
Accepted: 18 April 2019
Published: 07 May 2019

Citation:
Gómez-Boronat M, Isorna E, Armirotti A, Delgado MJ, Piomelli D and de Pedro N (2019) Diurnal Profiles of N-Acylethanolamines in Goldfish Brain and Gastrointestinal Tract: Possible Role of Feeding. Front. Neurosci. 13:450. doi: 10.3389/fnins.2019.00450

N-acylethanolamines (NAEs) are a family of endogenous lipid signaling molecules that are involved in regulation of energy homeostasis in vertebrates with a putative role on circadian system. The aim of this work was to study the existence of daily fluctuations in components of NAEs system and their possible dependence on food intake. Specifically, we analyzed the content of oleoylethanolamide (OEA), palmitoylethanolamide (PEA), stearoylethanolamide (SEA), their precursors (NAPEs), as well as the expression of *nape-pld* (NAEs synthesis enzyme), *faah* (NAEs degradation enzyme), and *pparα* (NAEs receptor) in gastrointestinal and brain tissues of goldfish (*Carassius auratus*) throughout a 24-h cycle. Daily profiles of *bmal1a* and *rev-erbα* expression in gastrointestinal tissues were also quantified because these clock genes are also involved in lipid metabolism, are PPAR-targets in mammals, and could be a link between NAEs and circadian system in fish. Gastrointestinal levels of NAEs exhibited daily fluctuations, with a pronounced and rapid postprandial increase, the increment being likely caused by food intake as it is not present in fasted animals. Such periprandial differences were not found in brain, supporting that NAEs mobilization occurs in a tissue-specific manner and suggesting that these three NAEs could be acting as peripheral satiety signals. The abundance of *pparα* mRNA displayed a daily rhythm in the intestine and the liver, suggesting a possible rhythmicity in the NAEs functionality. The increment of *pparα* expression during the rest phase can be related with its role stimulating lipid catabolism to obtain energy during the fasting state of the animals. In addition, the clock genes *bmal1a* and *rev-erbα* also showed daily rhythms, with a *bmal1a* increment after feeding, supporting its role as a lipogenic factor. In summary, our data show the existence of all components of NAEs system in fish (OEA, PEA, SEA, precursors, synthesis and degradation enzymes, and the receptor PPARα), supporting the involvement of NAEs as peripheral satiety signals.

Keywords: OEA, PEA, SEA, acylethanolamides, PPARα, food intake, rhythms, fish

INTRODUCTION

Acylethanolamides or *N*-acylethanolamines (NAEs) are a family of endogenous bioactive lipid molecules present in animal, plant, as well as in prokaryotic cells (Hansen and Vana, 2018), which play a key role in feeding regulation in vertebrates (Borrelli and Izzo, 2009; Hansen, 2014; Kleberg et al., 2014; Romano et al., 2015). They consist on a fatty acid linked by an amide bound to an ethanolamine and are classified based on the number of carbons and degree of saturation of their acyl chain. Major NAEs in mammalian tissues comprise oleoylethanolamide (N-oleoylethanolamine, OEA), palmitoylethanolamide (*N*-palmitoylethanolamine, PEA) and stearoylethanolamide (N-stearoylethanolamine, SEA), and several other quantitative minor species including anandamide (*N*-arachidonoylethanolamine, AEA; Tsuboi et al., 2013).

Endogenous levels of NAEs are mainly regulated by enzymes responsible for their formation and degradation. Biosynthesis of NAEs is an "on demand" process with two major steps, the formation of *N*-acylphosphatidylethanolamines (NAPEs) from their phospholipid precursors through a Ca^{2+}-dependent N-acyltransferase (NAT) activity and the conversion of NAPEs to NAEs via several pathways (Tsuboi et al., 2013; Rahman et al., 2014). In animals, most NAEs result from a NAPE hydrolysis catalyzed in a single enzymatic step by a specific membrane-bound phospholipase D, namely NAPE-PLD, although other multi-step pathways have been described (Borrelli and Izzo, 2009; Tsuboi et al., 2013; Ueda et al., 2013; Inoue et al., 2017). The generated NAEs are rapidly catabolized by fatty acid amide hydrolase (FAAH) to their corresponding free fatty acids and ethanolamine. FAAH is localized in endoplasmic reticulum and functions as a general inactivating enzyme for all NAEs in mammals, with highest activity in liver, small intestine and brain (Ueda et al., 2013; Kleberg et al., 2014; Hansen and Vana, 2018). Moreover, a NAE-hydrolyzing acid amidase (NAAA) localized in lysosomes contributes to NAEs catabolism, and preferentially hydrolyzes PEA over the other NAEs (Ueda et al., 2013).

These NAEs can interact with different receptors which are involved in many physiological processes, playing an important role in the regulation of energy homeostasis (Borrelli and Izzo, 2009; Hansen, 2014; Kleberg et al., 2014; Romano et al., 2015). The most studied NAE in mammals is OEA, which acts as an anorexigenic signal and promotes fat catabolism (Bowen et al., 2017; Sihag and Jones, 2018). In goldfish, OEA also reduces food intake and body weight, and is involved in lipid and glucose metabolism (Tinoco et al., 2014; Gómez-Boronat et al., 2016). A food intake decrease was also observed after exogenous administration of PEA or SEA in the only vertebrates studied to date, rats and mice (Rodríguez de Fonseca et al., 2001; Terrazzino et al., 2004). These functions seem to be mediated via activation of the transcription factor peroxisome proliferator-activated receptor alpha (PPARα), although OEA and PEA also bind other receptors, such as G protein-coupled receptor GPR119 and a transient receptor potential vanilloid type 1 (TRPV1; Hansen, 2010; Kleberg et al., 2014).

Fed and fasted states modulate NAEs production in vertebrates. Intestinal levels of OEA are decreased by food deprivation and increased upon refeeding in rodents (Rodríguez de Fonseca et al., 2001; Piomelli, 2013; Bowen et al., 2017) and goldfish (Tinoco et al., 2014). A similar feeding-induced PEA mobilization in small intestine, without modifications in SEA, has also been described in rats (Petersen et al., 2006); although another study reported that food deprivation does not change the duodenal or jejunal content of both PEA and SEA in this species (Fu et al., 2007). A postprandial OEA, PEA, and SEA increment has also been described in small intestine of Burmese python (*Python molurus*), the only reptile species so far studied (Astarita et al., 2006). These postprandial variations seem to occur in a tissue-specific manner, since changes in OEA and PEA levels in response to feeding were not observed in other peripheral tissues and brain structures in rats (Fu et al., 2007; Izzo et al., 2010).

Because feeding is usually a rhythmic behavior, the existence of daily fluctuations in some feeding regulators has been reported (Bechtold and Loudon, 2013; Isorna et al., 2017). However, only few studies have investigated the daily rhythmicity in the NAEs system and its possible interaction with the circadian system, with no consistent results. Diurnal fluctuations of endogenous levels of NAEs have been found in brain: while in the cerebrospinal fluid, OEA and PEA concentrations increased during the light-on period; in pons, hippocampus and hypothalamus, these NAEs increased during the dark phase in rat (Murillo-Rodriguez et al., 2006). However, OEA levels in various brain regions of mice did not change between 11:30 a.m. and 11:30 p.m. (Guijarro et al., 2010). In gastrointestinal tissues, OEA levels display diurnal fluctuations in rodents, being higher during the daytime, when animals are satiated, and lower during the night, when they are awaked and actively feeding (Fu et al., 2003; LoVerme et al., 2005; Guijarro et al., 2010). No differences were found in other components of NAEs system, such as PEA, NAPEs and activity of the enzymes NAPE-PLD and FAAH at the midpoint of the light and dark phase in mice jejunum (Guijarro et al., 2010). FAAH activity was also similar in various brain regions, with a decline only in cerebellum (Glaser and Kaczocha, 2009), striatum, and hippocampus (Valenti et al., 2004) at midnight. Although FAAH K_m and V_{max} are affected by time of the day in some brain regions of mice, none of the data support a primary role for FAAH in the circadian regulation of the brain NAEs (Liedhegner et al., 2014). To date, the clearest link between NAEs and the circadian system is the fact that their main receptor, PPARα, directly regulates the transcription of BMAL1 and REV-ERBα, two core clock genes, which possess a PPAR response element (PPRE) in their promoters (Charoensuksai and Xu, 2010; Chen and Yang, 2014). In addition, PPARα is also a direct target gene of the heterodimer CLOCK/BMAL1, a key component of the molecular clock which drive rhythms in target genes known as clock-controlled genes. Thus, PPARα is considered an output gene and shows daily rhythmic expression in a variety of tissues in mammals (Yang et al., 2006; Chen et al., 2010). This interaction between OEA and the circadian system has also been suggested in fish, since hepatic expression of *bmal1a* increases after OEA treatment

in goldfish (Gómez-Boronat et al., 2016) and the expression of *pparα* is rhythmic in gilthead sea bream (*Sparus auratus*; Paredes et al., 2014) and zebrafish (*Danio rerio*; Paredes et al., 2015). Apart from these data, there is no evidence in fish on the possible daily rhythmicity in OEA and other NAEs.

The aim of this work was to study the existence of daily fluctuations in the NAEs system components and their possible regulation by food intake in fish. Specifically, we quantified the content of OEA, PEA, SEA, and their precursors (NAPEs) in central and peripheral tissues of goldfish throughout a 24-h cycle. The daily pattern of expression in gastrointestinal tissues of *napepld* (NAEs synthesis enzyme), *faah* (NAEs degradation enzyme), and *pparα* (NAEs receptor) was also measured. Moreover, the enzymatic activity of FAAH was quantified in anterior intestine and hypothalamus. Finally, the daily rhythmic expression of the clock genes *bmal1a* and *rev-erbα* was analyzed in gastrointestinal tissues to investigate a possible interaction between NAEs and the circadian system.

MATERIALS AND METHODS

Animals and Housing

Goldfish with a body mass (bm) of 23 ± 6 g were obtained from a local commercial supplier (ICA, Madrid, Spain). Fish were housed in 60 l aquaria with filtered fresh water (21 ± 1°C) and continuous aeration and maintained under a 12 h light: 12 h darkness (12L:12D) photoperiod (lights on at 8 a.m., considered as *zeitgeber* time 0-ZT0). The aquaria walls were covered with opaque paper to minimize external interferences during the experiment. Fish were fed (1% bm) once daily at 10 a.m. (ZT2) with commercial dry pellets (32.1% crude protein, 5% crude fat, 1.9% crude fiber, 6.8% crude ash, 5.1% water, and the rest nitrogen free extract; Sera Pond, Heinsberg, Germany). Animals were maintained under these conditions for 1 month. The experiments described comply with the Guidelines of the European Union Council (UE63/2010) and the Spanish Government (RD53/2013) for the use of animals in research and were approved by the Animal Experimentation Committee of Complutense University (O.H.-UCM-25-2014) and the Community of Madrid (PROEX 107/14).

Experimental Design

Goldfish (n = 49) were sampled throughout a 24-h cycle each 4 h (n = 7 per sampling point; ZT3, ZT7, ZT11, ZT15, ZT19, ZT23, and ZT3 of next day -ZT3b). Food was offered as scheduled (ZT2) the first day of the experiment, but not the second day before last sampling point (ZT3b). Thus, the possible effect of food intake on the NAEs system was tested by comparing fish sampled at the same time but 1-h postprandial (ZT3) or 25-h fasting (ZT3b). In each sampling point, animals were sacrificed by anesthetic overdose (tricaine methanesulfonate, MS-222, 0.28 g/l; Sigma-Aldrich, Madrid, Spain) followed by spinal cord section. Tissue were quickly dissected: initial and final segments of intestinal bulb, anterior intestine in two sections, liver in three aliquots, and central tissues (hypothalamus and telencephalon) as a whole. All samples were rapidly frozen in liquid nitrogen and immediately stored at −80°C until analysis.

Determination of Tissue Content of NAEs and NAPEs

A longitudinal half of the final segment of the intestinal bulb, a transversal half of the initial segment of the anterior intestine, and one liver aliquot were weighed (20–30 mg) as well as a longitudinal half of both hypothalamus and telencephalon (5–10 mg). Samples were homogenized in 1 ml of methanol (Thermo Fisher Scientific, Milan, Italy) containing the following deuterated internal standards (IS): OEA–d_4 (100 nM), PEA–d_4 (100 nM), SEA–d_3 (100 nM), and C17:0 NAPE (25 nM) (Cayman Chemical, Ann Arbor, MI, United States). Then, this solution was mixed with 2 v of chloroform (Thermo Fisher Scientific) and 1 v of water. Organic phase was collected, dried under nitrogen atmosphere, and fractioned by open-bed silica gel column chromatography, as previously described (Cadas et al., 1997; Tinoco et al., 2014). Briefly, the lipid extracts were reconstituted in chloroform and loaded onto small columns packed with Silica Gel G (60 Å 230–400 Mesh ASTM; Whatman, Clifton, NJ, United States). NAEs and NAPEs were eluted with a methanol:chloroform solution (1:9 and 1:1, respectively). Both eluates were again dried under nitrogen atmosphere and, subsequently, NAEs were reconstituted in 75 µl and NAPEs in 100 µl of methanol:chloroform (9:1). Samples were then analyzed by UPLC–MS/MS on a Xevo–TQ triple quadruple mass spectrometer coupled with an UPLC (ultra-performance liquid chromatography) system (Waters Inc., Milford, PA, United States). NAEs and its deuterated analogs were loaded on a reversed phase BEH C18 column (50 × 2.1 mm inner diameter, 1.7 µm particle size, maintained at 45°C; Waters Inc.) operated at a constant flow rate of 0.5 ml/min. The mobile phase consisted of 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. A step gradient program was developed for the best separation of all metabolites: 0–0.5 min 20% B and 0.5–3.0 min 100% B. The column was then reconditioned to 20% B for 0.5 min. The total run time for analysis was 3.5 min and the injection volume 5 µl. For analysis of NAPEs of PEA and SEA and their deuterated analogs, a reversed phase T3 column (50 × 2.1 mm inner diameter, 1.8 µm particle size, maintained at 50°C; Waters Inc.) was used with a constant flow rate of 0.4 ml/min. The mobile phase consisted of 10 mM ammonium formate in acetonitrile:water (60:40) as solvent A and 10 mM ammonium formate in acetonitrile:isopropanol (10:90) as solvent B. A step gradient program was developed for the best separation of all metabolites: 0–0.5 min 50% B, 0.5–3.5 min 50 to 100% B, 3.5–4.5 min 100% B, and 4.5–5.0 min 100 to 50% B. The column was then reconditioned to 50% B for 1 min. The total run time for analysis was 6 min and the injection volume 5 µl. Lastly, conditions for analysis of NAPEs of OEA were the same as above for the other NAPEs with little modifications: constant flow rate of 0.35 ml/min; a step gradient of 0–0.5 min 30% B, 0.5–6.0 min 30 to 100% B, 6.0–7.0 min 100% B, 7.0–7.1 min 100 to 50% B, and reconditioned column to 30% B for 1.9 min; and total run time for analysis of 9 min. For both NAEs and NAPEs,

the mass spectrometer was operated in the positive ESI mode, the capillary voltage was set at 3 kV, the cone voltage was set at 20 V for all transitions, and analytes were quantified by multiple reactions monitoring (MRM). The complete panel of source parameters and MRM transitions are reported in the datasheet of the Supplementary Material (**Supplementary Tables S1**, **S2**). The source temperature was set to 120°C. Desolvation gas and cone gas (N_2) flows were set to 800 and 50 l/h, respectively. Desolvation temperature was set to 450°C. Data were acquired by MassLynx software and quantified by TargetLynx software. Calibration curves (0.1 to 100 nM range for all compounds) were constructed by plotting the analyte to IS peak areas ratio versus the corresponding analyte concentration using weighted (1/×) least square regression analysis.

Determination of FAAH Activity

The initial segment of the anterior intestine (the other transversal half) and the other longitudinal half of hypothalamus were weighed, homogenized in ice-cold Tris–HCl buffer (20 mM, pH 7.4) containing 0.32 M sucrose, and centrifuged at 1000 × *g* for 10 min at 4°C. Supernatants were collected and protein concentrations determined using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, United States). To measure FAAH activity, 0.5 ml of Tris–HCl buffer (50 mM, pH 7.4) containing fatty acid-free bovine serum albumin (0.05%), tissue homogenates (50 μg of protein), 10 μM AEA and AEA-(ethanolamine-^{3}H) (20,000 cpm, specific activity 60 Ci/mmol; American Radiolabeled Chemicals, St Louis, MO, United States) were incubated at 37°C for 30 min. Reactions were stopped with 1 ml methanol:chloroform (1:1), centrifuged at 1400 × *g* for 10 min at 4°C, and radioactivity was measured in the aqueous phase by liquid scintillation counting in MicroBeta LumiJET system (Perkin Elmer Inc., Waltham, MA, United States).

Gene Expression Analysis

Total RNA from the initial segment of the intestinal bulb (3 mm), the distal segment of the anterior intestine (5 mm), and the other liver aliquot was isolated using TRI® Reagent (Sigma-Aldrich) and treated with RQ1 RNase-Free DNase (Promega, Madison, United States) according to the manufacturer's instructions. Then, an aliquot of total RNA (0.1 μg of intestinal bulb and anterior intestine, or 0.3 μg of liver) was reverse transcribed into cDNA in a 25 μl reaction volume using random primers (Invitrogen, Carlsbad, United States), RNase inhibitor (Promega) and SuperScript II Reverse Transcriptase (Invitrogen). The reverse transcription reaction conditions consisted of an initial step at 25°C for 10 min, an extension at 42°C for 50 min, and a denaturalization step at 70°C for 15 min. Real-Time quantitative PCRs (RT-qPCRs) were carried out by duplicate in a CFX96™ Real-Time System (Bio-Rad Laboratories, Hercules, United States), using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) in a 96-well plate loaded with 1 μl of cDNA and a final concentration of 0.5 μM of each forward and reverse primers in a final volume of 10 μl. Each PCR run also included a 4-point serial standard curve, non-retrotranscribed RNA (as positive control) and water (as negative control). The RT-qPCR cycling conditions consisted of an initial denaturation at 95°C for 30 s and 40 cycles of a two-step amplification program (95°C for 5 s and 60°C for 30 s). A melting curve was systematically monitored (temperature gradient at 0.5°C/5 s from 70 to 90°C) at the end of each run to confirm the specificity of the amplification reaction. The Gene Data Bank reference numbers and the primers (Sigma-Aldrich) sequences employed for target genes (*nape-pld*, *faah* *ppar*α, *bmal1a*, and *rev-erb*α) and the reference gene (*ef-1*α) are shown in **Table 1**. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression (fold change). Data obtained were normalized to the group with the lowest expression in each gene.

TABLE 1 | Accession numbers and primers sequences of the genes employed in the RT-qPCR assays.

Gene	Accession number	Primer sequence 5′ → 3′		Product (bp)
nape-pld	MH638307	Forward	TGCTCGCTTTGGTTCCAGT	115
		Reverse	AATGCAGTTTCCCACCCAC	
faah	HM231167.1	Forward	TTGGAGGAGGAGGCTCTTTG	115
		Reverse	CCACTGCAATGAGGATAAGTGC	
*ppar*α	AY198322.1	Forward	CCATCCCGACAACGAGTTCC	121
		Reverse	CAGCGACGTGTCTTCTGTCT	
bmal1a	KF840401	Forward	AGATTCTGTTCGTCTCGGAG	161
		Reverse	ATCGATGAGTCGTTCCCGTG	
*rev-erb*α	KU242427.1	Forward	CGTTCATCTCAGGCACCACT	166
		Reverse	AACTGACCTGCAGACACCAG	
*ef-1*α	AB056104	Forward	CCCTGGCCACAGAGATTTCA	101
		Reverse	CAGCCTCGAACTCACCAACA	

*Nape-pld, N-acyl phosphatidylethanolamine-specific phospholipase D; faah, fatty acid amidohydrolase; ppar*α*, peroxisome proliferator-activated receptor* α*; bmal1a, brain and muscle ARNT-like 1a; rev-erb*α*, nuclear receptor subfamily 1 group D member 1 (NR1D1); ef-1*α*, elongation factor-1*α*.*

Data Analysis

All studied parameters were first analyzed by One-way ANOVA followed by the *post hoc* Student–Newman–Keuls (SNK) test (using SigmaPlot 12.0 statistics package). When necessary, data were transformed to logarithmic or square root scale to normalize and to obtain homoscedasticity. In addition, a Student *t*-test was performed to compare data from 1 h postprandial (sampling point ZT3) and 25 h fasting (ZT3b). A probability level of $p < 0.05$ was considered statistically significant in all tests. Furthermore, the existence of daily (24-h) rhythms were determined by Cosinor analysis fitting the data to a sinusoidal function by the least squares method (Duggleby, 1981). The formula used was $f(t) = M + A * \cos(t\pi/12 - \Phi\pi/12)$, where $f(t)$ was the gene expression level at a given time, the mesor (M) is the mean value, A is the sinusoidal amplitude of oscillation, t is time in hours, and Φ is the acrophase (time of peak expression). Non-linear regression allows the estimation of M, A, and Φ, and their standard error (SE), being the SE based on the residual sum of squares in the least-squares fit (Duggleby, 1981; Delgado et al., 1993). Significance of Cosinor analysis was defined by the noise/signal of amplitude calculated from the ratio $SE(A)/A$ (Nisembaum et al., 2012). Data were considered to display a daily rhythm if it had both $p < 0.05$ by ANOVA and $SE(A)/A < 0.3$ by Cosinor analysis.

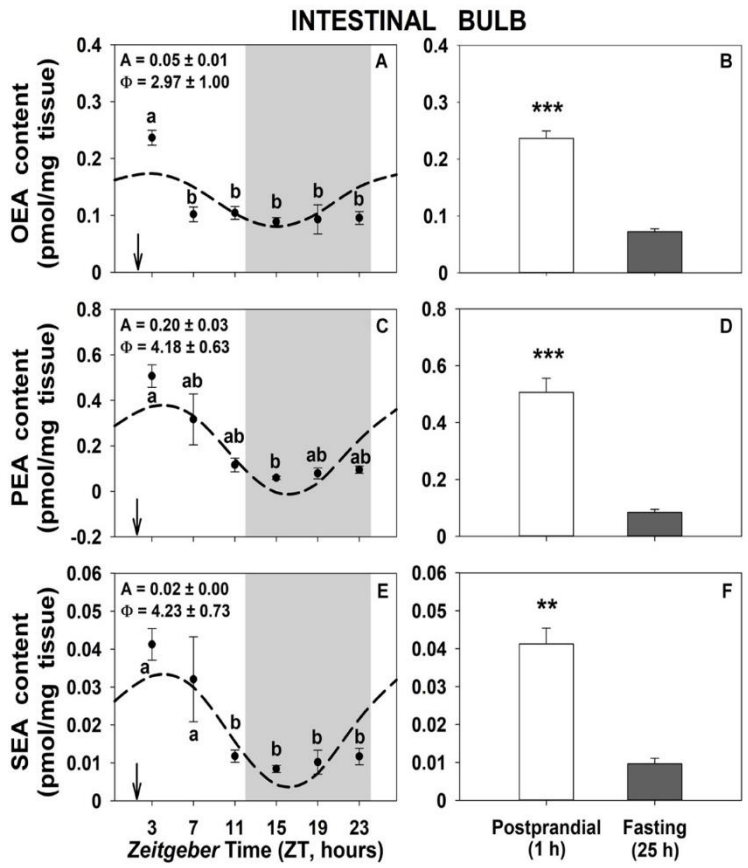


FIGURE 1 | Daily variations of NAEs (OEA, PEA, and SEA) content in intestinal bulb of goldfish. **(A,C,E)**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). *Different letters* indicate significant differences among groups (ANOVA, $p < 0.05$ and SNK *post hoc* test). Dashed lines represent periodic sinusoidal functions when rhythms are significant [Cosinor, $SE(A)/A < 0.3$; and ANOVA, $p < 0.05$]. Amplitude (A, fold change) and acrophase (Φ, h) values of the rhythms are shown in the top-left of the graphs. **(B,D,F)**, comparison between 1 h postprandial and 25 h fasting. *Asterisks* indicate significant differences by Student *t*-test analysis (**$p < 0.01$, ***$p < 0.001$). Data are shown as mean ± SEM ($n = 6$–7).

RESULTS

Daily patterns of OEA, PEA, and SEA levels in intestinal bulb of goldfish are shown in **Figure 1**. All three NAEs displayed significant rhythms with low amplitudes and the acrophase (time of the day with maximum levels) 3–4 h after mealtime (**Figures 1A,C,E**). The content of the three NAEs was 3–4 fold higher in the 1-h postprandial fishes than in 25-h fasting ones (**Figures 1B,D,F**). Similar results were obtained in the other two gastrointestinal tissues (**Supplementary Figures S1**, **S3**), being rhythmic PEA and SEA in the anterior intestine and OEA in the liver. As for intestinal bulb, the content of NAEs was higher in the 1-h postprandial than in 25-h fasting fish in both peripheral tissues, although the trend not was statistically significant in the case of SEA in the liver (**Supplementary Figure S3F**).

Regarding the precursors of NAEs, the daily profiles of NAPEs corresponding to each NAE in intestinal bulb of goldfish are shown in **Figure 2**. We can observe daily variations in the content of NAPE of OEA and PEA in intestinal bulb with the maximum levels occurring during the day time and the lowest in the middle of the night (**Figures 2A,C,E**). Only the NAPE of OEA displayed a significant rhythm (**Figure 2A**). On the other hand, 25-h fasting did not modify the total amount of NAPEs respect to 1-h postprandial (**Figures 2B,D,F**). In the other two gastrointestinal tissues, the anterior intestine and the liver (**Supplementary Figures S2**, **S4**, respectively), no significant daily oscillations of NAPEs were found and only the PEA-NAPE showed a significant higher content in the 1-h postprandial compared to 25-h fasting animals.

Daily patterns of NAEs levels in the hypothalamus are shown in **Figure 3**. Contrary to gastrointestinal tissues, no significant differences were found neither throughout the 24-h cycle (**Figures 3A,C,E**) nor when 1-h postprandial and 25-h fasting animals were compared (**Figures 3B,D,F**). Similar results were obtained in the other studied brain tissue, the telencephalon (**Supplementary Figure S5**), except for SEA that showed daily oscillations with the lowest content at the beginning of the

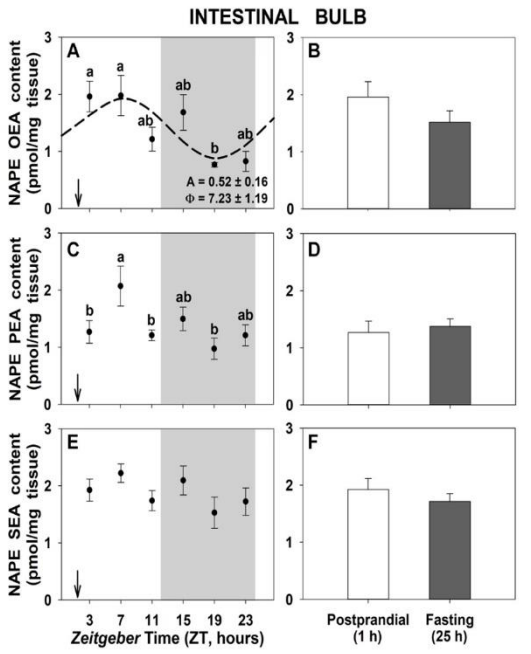


FIGURE 2 | Daily variations of NAPEs content in intestinal bulb of goldfish. **(A,C,E)**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). *Different letters* indicate significant differences among groups (ANOVA, $p < 0.05$ and SNK *post hoc* test). Dashed line represents periodic sinusoidal function when rhythm is significant [Cosinor, $SE(A)/A < 0.3$; and ANOVA, $p < 0.05$]. Amplitude (A, fold change) and acrophase (Φ, h) values of the rhythm are shown in the bottom-right of the graphs. **(B,D,F)**, comparison between 1 h postprandial and 25 h fasting. Data are shown as mean ± SEM ($n = 6–7$).

dark phase, although rhythmicity did not reach the threshold of significance (**Supplementary Figure S5E**).

Figure 4 shows the daily profiles of the NAPEs corresponding to each NAE in the hypothalamus of goldfish. No significant oscillations were obtained throughout the 24-h cycle (**Figures 4A,C,E**). However, significant differences between 1-h postprandial and 25-h fasting were noticed for all NAPEs in this encephalic tissue (**Figures 4B,D,F**). While hypothalamic levels of NAPE of OEA was increased by fasting, both NAPEs of PEA and SEA were decreased. Obtained results in the other central tissue, the telencephalon (**Supplementary Figure S6**), showed daily oscillations in all NAPEs with only a significant 24-h rhythm in the NAPE of OEA (**Supplementary Figures S6A,C,E**), but did not exhibit feeding-induced changes in the levels of any studied NAPEs (**Supplementary Figures S6B,D,F**).

The enzymatic activity of FAAH, the degradation enzyme of NAEs, in anterior intestine and hypothalamus of goldfish is shown in **Figure 5**. There are no daily changes in none of the studied tissues (**Figures 5A,C**). Instead, a significant threefold increase were noted in 1-h postprandial fish respect to 25-h fasting in the anterior intestine, while FAAH activity in hypothalamus remained unchanged with feeding (**Figures 5B,D**, respectively).

The mRNA abundance of *nape-pld* and *faah* genes (which codify for the NAEs synthesis and degradation enzymes, respectively) in intestinal bulb presented significant daily rhythms (**Figure 6**), with low amplitudes, and no differences were found between 1 h-posprandrial and 25 h fasting (data not shown). The acrophase of the *nape-pld* gene took place in the interphase dark-light, 2 h before the mealtime, while the acrophase of *faah* gene took place around ZT4, 2 h after the mealtime. A similar pattern was observed for *nape-pld* in liver but not in the anterior intestine (**Supplementary Figures S7A,C**) and for *faah* in both anterior intestine and liver (**Supplementary Figures S7B,D**), although such daily differences were not associated with significant rhythms.

Daily significant expression rhythms of the NAEs receptor (*pparα*) and of the two studied clock genes (*bmal1a* and *rev-erbα*) were found in the intestinal bulb of goldfish (**Figure 7**). The acrophase of *pparα* (**Figure 7A**) took place at ZT1, 1 h before mealtime and 1 h after the onset of the light, while the acrophase of *bmal1a* (**Figure 7B**) took place around ZT8, 6 h after mealtime. The acrophase of *rev-erbα* (**Figure 7C**) occurs at ZT17, almost in the middle of the scotophase. With regards to the other two peripheral tissues, the anterior intestine and the liver (**Supplementary Figure S8**), the circadian rhythms were maintained as for the intestinal bulb, with comparable acrophases and amplitudes.

DISCUSSION

Our results show for the first time in a fish species the existence of all components of NAEs system: OEA, PEA, SEA, their precursors, enzymes of synthesis and degradation, and the receptor PPARα. Endogenous levels of NAEs and NAPEs found in gastrointestinal and brain tissues in goldfish are similar to that previously reported in other vertebrates, although very few species have been studied (Astarita et al., 2006; Murillo-Rodriguez et al., 2006; Fu et al., 2007; Guijarro et al., 2010; Liedhegner et al., 2014). These data suggest that these bioactive lipids may be widespread across vertebrate groups.

NAEs (OEA, PEA, and SEA) exhibit daily variations in the goldfish gastrointestinal tissues, which seemed to follow daily rhythmic patterns, being mainly driven by food intake. In fact, the most interesting result of this study is a pronounced and rapid postprandial increase in the content of the three NAEs analyzed in intestinal bulb, anterior intestine, and liver of goldfish, compared to levels found in 25-h fasting fish. These results agree with the OEA formation promoted by feeding in the small intestine previously found in mammals, reptiles, and goldfish (Astarita et al., 2006; Piomelli, 2013; Tinoco et al., 2014; Bowen et al., 2017). Both rat and Burmese python also exhibit these fasting/refeeding-induced changes in the intestinal content of PEA and SEA (Astarita et al., 2006; Petersen et al., 2006; Diep et al., 2011). These periprandial fluctuations in the gastrointestinal content of NAEs suggest that this family of bioactive lipids may contribute to the regulation of feeding behavior in vertebrates, possibly acting as satiety signals, since intestinal levels are elevated in the post-ingestive

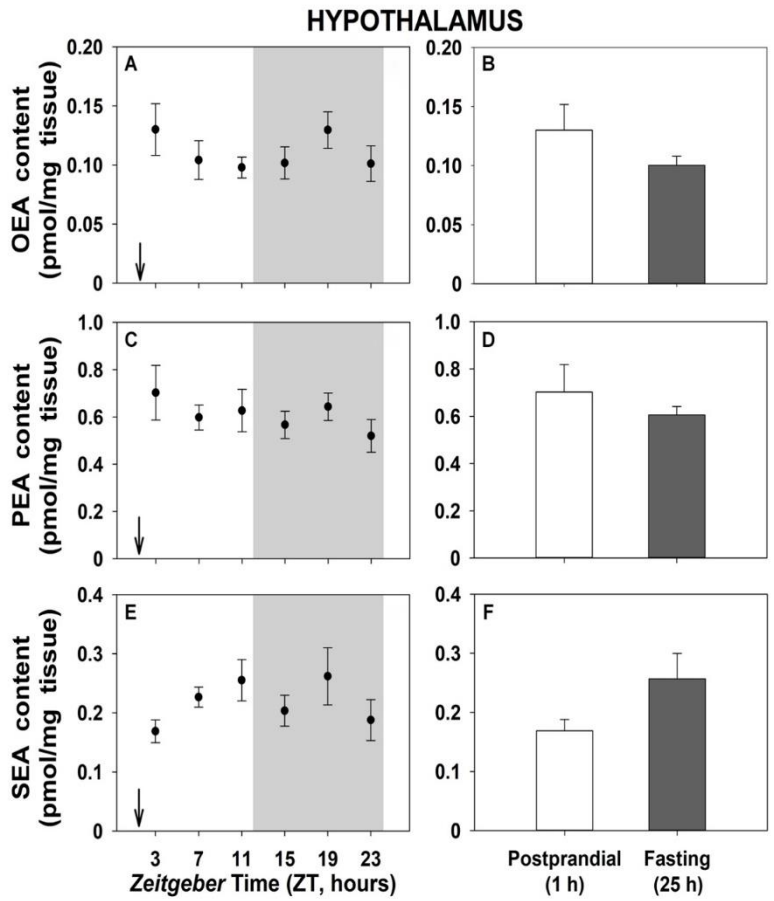


FIGURE 3 | Daily variations of NAEs (OEA, PEA, and SEA) content in hypothalamus of goldfish. **(A,C,E)**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). **(B,D,F)**, comparison between 1 h postprandial and 25 h fasting. Data are shown as mean ± SEM (n = 6–7).

state. Pharmacological acute studies in rodents support this idea, demonstrating anorectic effects of OEA, PEA and SEA in rats and mice, which are peripherally mediated (Rodríguez de Fonseca et al., 2001; Terrazzino et al., 2004; Piomelli, 2013). Similar results have also been found in goldfish, where a reduction in food intake was produced after intraperitoneal administration of OEA (Tinoco et al., 2014). Thus, all results support that NAEs are involved in feeding regulation, acting as short-term anorectic signals.

The postprandial increase of NAEs in intestinal tissues may be due to an increase of their precursors and/or changes in the activity of enzymes involved in their synthesis and degradation (Fu et al., 2007; Bowen et al., 2017). Some studies in mammals have suggested that NAEs levels are regulated in intestinal tissue in parallel with the formation of their precursor molecules, the NAPEs (Petersen et al., 2006; Fu et al., 2007; Gillum et al., 2008). In addition, Fu et al. (2007) found that feeding stimulates OEA mobilization in duodenum and jejunum by increasing activity and expression of NAPE-PLD. However, most data in the present study indicate that there are no feeding-related differences in the gastrointestinal content of NAPEs nor relative mRNA expression of *nape-pld*, even though significant changes in NAEs levels were observed during fasting/feeding cycles. Some hypotheses could explain this lack of differences in precursors and synthesis enzyme in fish. On one hand, it should also be taken into account that, in addition to the direct hydrolysis from NAPEs to NAEs by NAPE-PLD, other multistep pathways of NAEs formation exist (Hussain et al., 2017; Inoue et al., 2017). These alternative pathways involve intermediate molecules, such as glycerophospho-NAE, lyso-N-acyl-phosphatidylethanolamine, or phospho-*N*-acylethanolamine, and a possible decrease of these intermediates could explain the increase of NAEs levels observed in gastrointestinal tissues in goldfish, without feeding-related modifications in levels of NAPEs and the NAPE-PLD enzyme. In addition, Lin et al. (2018) demonstrated that dietary fatty acids can modulate tissue NAEs levels in the absence of NAPE-PLD, which suggest that NAPE-PLD is not necessary for NAEs synthesis, thereby highlighting the important role of alternative pathways in maintaining NAEs levels. Other possibility is that the NAEs regulation by feeding occurs at level of the degradation

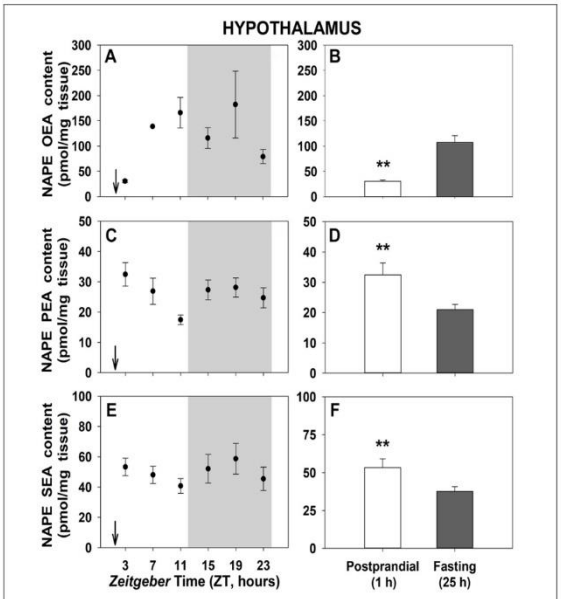


FIGURE 4 | Daily variations of NAPEs content in hypothalamus of goldfish. **(A,C,E)**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). **(B,D,F)**, comparison between 1 h postprandial and 25 h fasting. *Asterisks* indicate significant differences by Student *t*-test analysis (**$p < 0.01$). Data are shown as mean ± SEM ($n = 6–7$).

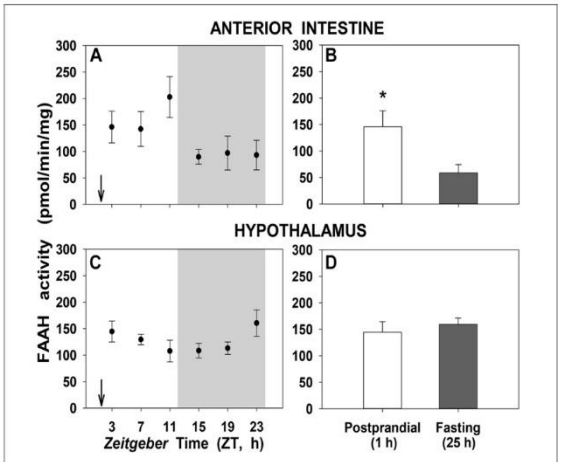


FIGURE 5 | Daily variations of FAAH activity in anterior intestine **(A,B)** and hypothalamus **(C,D)** of goldfish. **(A,C)**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). **(B,D)**, comparison between 1 h postprandial and 25 h fasting. *Asterisks* indicate significant differences by Student *t*-test analysis (*$p < 0.05$). Data are shown as mean ± SEM ($n = 6–7$).

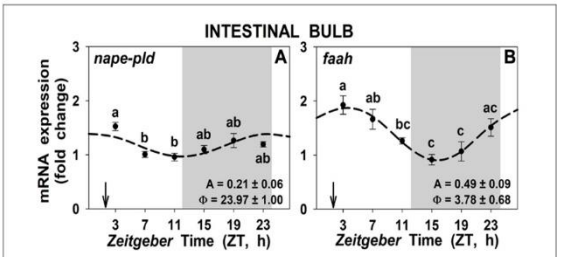


FIGURE 6 | Daily rhythms of *nape-pld* **(A)** and *faah* **(B)** expression in intestinal bulb of goldfish. Gray area indicates the dark phase and arrows the feeding time (ZT2). Data are shown as mean ± SEM ($n = 6–7$) in relative units ($2^{-\Delta\Delta Ct}$ method). *Different letters* indicate significant differences among groups (ANOVA, $p < 0.05$ and SNK *post hoc* test). Dashed lines represent periodic sinusoidal functions when rhythms are significant [Cosinor, $SE(A)/A < 0.3$; and ANOVA, $p < 0.05$]. Amplitude (A, fold change) and acrophase (Φ, h) values of the rhythms are shown in the bottom-right of the graphs.

enzyme, since a decrease in FAAH activity and expression in rodents' intestine was found after feeding (Fu et al., 2007), being responsible, at least in part, of feeding-induced OEA increase. However, this expected negative correlation between NAEs concentration and FAAH was not found in goldfish tissues. Other studies in mammals have indicated that FAAH activity has a small contribution in NAEs levels, suggesting the existence of other amidases, such as NAAA, also responsible for NAEs metabolism (Borrelli and Izzo, 2009; Liedhegner et al., 2014; Bowen et al., 2017). Thus, other forms of amidase not yet characterized in fish could also contribute to the postprandially NAEs-increased levels in goldfish. The postfeeding increase in the expression and activity of FAAH in some gastrointestinal tissues of goldfish could be a physiological response to the rise in NAEs levels due to the upregulation of any enzyme involved in the formation of NAEs, as it has been previously suggested in rats (Diep et al., 2011).

Less clear is the role of NAEs at central level and very few studies have examined daily changes in the brain content of NAEs. In the present study, the NAEs content in the goldfish hypothalamus and telencephalon did not display significant rhythms. Similar results were found in mice, where daily oscillations were not detected in hypothalamic content of OEA and PEA, although these NAEs exhibited diverse daily rhythms in other brain regions, such as cerebellum, amygdala, and hippocampus, suggesting that these daily changes in NAEs are brain region-specific (Liedhegner et al., 2014). Controversial results have been reported in rats. While no effect of daytime (photophase versus scotophase) was found in OEA content in various encephalic tissues in rats (cerebellum, hippocampus, hypothalamus, thalamus, cortex, striatum, and brainstem; Guijarro et al., 2010), diurnal variations of OEA and PEA were detected in pons, hippocampus, and hypothalamus in the same species (Murillo-Rodriguez et al., 2006). Independently of existence or not of daily modifications in the NAEs content in different encephalic tissues, it has been suggested that the brain content of these compounds seem to be feeding independent. Thus, OEA did not respond to food deprivation in different rat brain tissues, including structures involved in the control of feeding, such as hypothalamus, thalamus, cortex, striatum, and brainstem (Fu et al., 2007; Izzo et al., 2010). Similarly, no differences in NAEs content in hypothalamus and telencephalon were observed between fasting and feeding states

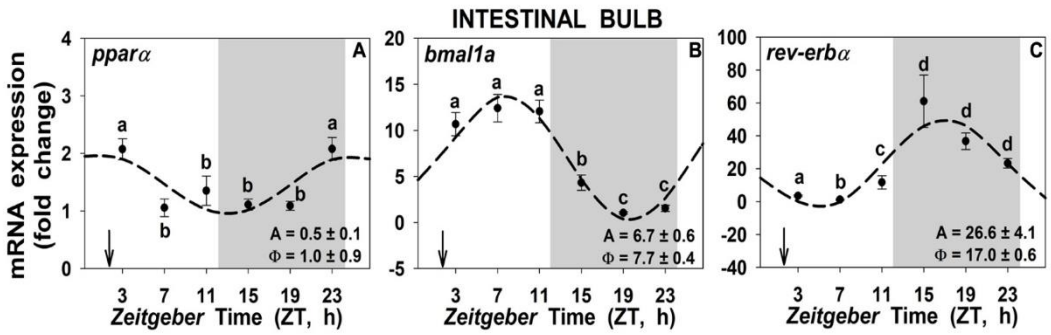


FIGURE 7 | Daily rhythms of *ppar*α **(A)**, *bmal1a* **(B)** and *rev-erb*α **(C)** expression in intestinal bulb of goldfish. Gray area indicates the dark phase and arrows the feeding time (ZT2). Data are shown as mean ± SEM (n = 6–7) in relative units ($2^{-\Delta\Delta Ct}$ method). *Different letters* indicate significant differences among groups (ANOVA, $p < 0.05$, and SNK *post hoc* test). Dashed lines represent periodic sinusoidal functions when rhythms are significant [Cosinor, $SE(A)/A < 0.3$; and ANOVA, $p < 0.05$]. Amplitude (A, fold change) and acrophase (Φ, h) values of the rhythms are shown in the bottom-right of the graphs.

in goldfish. This feeding-independent regulation of NAEs in the brain suggests that nutritional status could be regulating NAEs mobilization in a tissue-specific manner only at gastrointestinal level. In addition, it would support the above discussed idea that NAEs play a role in the feeding regulation at peripheral level. Nevertheless, it cannot be ruled out that NAEs play other physiological roles at the brain level, although they have not been investigated in fish yet.

Although NAPEs have been considered for a long time as simply phospholipid precursors of the NAEs, the increasing body of evidence in mammals has suggested that NAPEs also seem to be bioactive molecules that are involved in several physiological functions, without the involvement of NAEs (Coulon et al., 2012; Romano et al., 2015). Particularly, it has been demonstrated that hypothalamic administration of C16:0 NAPE (N-palmitoyl-phosphatidylethanolamine, the most abundant plasmatic NAPE) decreases food intake in rats, and its effect does not seem to be mediated by a NAPE metabolite (NAE) (Gillum et al., 2008; Wellner et al., 2011). In the present study, we have found postprandial changes in NAPEs at hypothalamic level in goldfish, which could suggest a possible involvement of these NAPEs in the central regulation of feeding. Although, because OEA-NAPE content decreases and PEA- and SEA-NAPE content increases after feeding, the interpretation of these results is difficult. Further studies must to be performed in order to clarify the exact role of NAPEs in fish brain and if they are signaling lipids able to control important biological functions on their own.

The expression of the NAEs receptor, *ppar*α, displayed a clear daily rhythm in all the studied gastrointestinal tissues, suggesting a possible rhythmicity in the functions of NAEs. In nocturnal rodents, *ppar*α expression in liver increases during the daytime, having its maximum at the beginning of the night (Yang et al., 2006; Chen et al., 2010; Wang et al., 2014). Our data indicate that in the diurnal-species goldfish, *ppar*α expression rises during the nighttime peaking in the early morning (1 h before feeding) in intestinal tissues and liver, similar to that reported in the liver of sea bream, another diurnal fish (Paredes et al., 2014). In both nocturnal and diurnal animals, the PPARα is upregulated during the rest phase, which coincides with the fasting state of the animals (Liu et al., 2014). During this fasting state, animals obtain energy from increasing the hepatic fatty acid oxidation with the synthesis of ketone bodies (Ribas-Latre and Eckel-Mahan, 2016). In fact, it has been demonstrated in mammals that PPARα stimulates fatty acid oxidation and lipid catabolism (Charoensuksai and Xu, 2010; Chen and Yang, 2014; Liu et al., 2014). In addition, *bmal1a* is also rhythmic increasing after feeding, supporting its role as a lipogenic factor in mammals (Shimba et al., 2005, 2011; Zhang et al., 2014), and also in goldfish (Gómez-Boronat et al., 2016). Moreover, PPARα has been largely proposed as a link between lipid metabolism and circadian system in mammals (Yang et al., 2006; Chen and Yang, 2014; Ribas-Latre and Eckel-Mahan, 2016), in which circadian rhythms of PPARα are essential for the temporal coordination of genes involved in energy and metabolic process (Charoensuksai and Xu, 2010; Chen and Yang, 2014; Ribas-Latre and Eckel-Mahan, 2016). Thus, it is widely known that PPARα directly regulates the transcription of *bmal1* and *rev-erb*α via binding to the peroxisome proliferator response element (PPRE) sites in their respective promoter regions. In addition, BMAL1 induces *ppar*α and *rev-erb*α by binding to an E-box rich region in their respective promoters (Canaple et al., 2006; Charoensuksai and Xu, 2010; Chen and Yang, 2014; Lecarpentier et al., 2014). In accordance with this regulatory loop, in the three gastrointestinal tissues of goldfish here analyzed (intestinal bulb, anterior intestine, and liver), the acrophases of *ppar*α and *bmal1a* are ∼8-h shifted, as previously reported in mammals (8–14-h shift; Canaple et al., 2006; Yang et al., 2006; Chen et al., 2010). The existence of the expected daily rhythms in all studied clock genes supports the idea that clocks in gastrointestinal tissues are functional.

In summary, the identification in goldfish of the NAEs system, including precursors, enzymes of synthesis and degradation and receptor, suggests that this endogenous system can be an important pathway for physiological functions as regulation of energy homeostasis in fish, as it is mammals. The gastrointestinal regulation of NAEs levels by the fed and fasted metabolic states supports that NAEs are involved in the feeding regulation, acting

as a peripheral satiety signal. In addition, the present results are in agreement with a putative role of PPARα as a functional link between the circadian clock and lipid metabolism in fish.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Guidelines of the European Union Council (UE63/2010) and the Spanish Government (RD53/2013). The protocol was approved by the Animal Experimentation Committee of Complutense University (O.H.-UCM-25-2014) and the Community of Madrid (PROEX 107/14).

AUTHOR CONTRIBUTIONS

MG-B, EI, MD, and NdP conceived and designed the experiments, carried out sampling, and interpreted findings. MG-B, EI, NdP, AA, and DP analyzed the samples. All authors drafted and revised the manuscript.

FUNDING

This study was supported by Spanish Ministerio de Economía y Competitividad project (MINECO, AGL2016-74857-C3-2-R). MG-B was a predoctoral fellow from Spanish MINECO (BES-2014-068103). The authors belong to the Fish Welfare and Stress Network (MINECO, AGL2016-81808-REDT).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00450/full#supplementary-material

REFERENCES

Astarita, G., Rourke, B. C., Andersen, J. B., Fu, J., Kim, J. H., Bennett, A. F., et al. (2006). Postprandial increase of oleoylethanolamide mobilization in small intestine of the *Burmese python* (*Python molurus*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290, R1407–R1412. doi: 10.1152/ajpregu.00664.2005

Bechtold, D. A., and Loudon, A. S. I. (2013). Hypothalamic clocks and rhythms in feeding behaviour. *Trends Neurosci.* 36, 74–82. doi: 10.1016/j.tins.2012.12.007

Borrelli, F., and Izzo, A. A. (2009). Role of acylethanolamides in the gastrointestinal tract with special reference to food intake and energy balance. *Best Pract. Res. Clin. Endocrinol. Metab.* 23, 33–49. doi: 10.1016/j.beem.2008.10.003

Bowen, K. J., Kris-Etherton, P. M., Shearer, G. C., West, S. G., Reddivari, L., and Jones, P. J. H. (2017). Oleic acid-derived oleoylethanolamide: a nutritional science perspective. *Prog. Lipid Res.* 67, 1–15. doi: 10.1016/j.plipres.2017.04.001

Cadas, H., di Tomaso, E., and Piomelli, D. (1997). Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain. *J. Neurosci.* 17, 1226–1242.

Canaple, L., Rambaud, J., Dkhissi-Benyahya, O., Rayet, B., Tan, N. S., Michalik, L., et al. (2006). Reciprocal regulation of brain and muscle Arnt-like protein 1 and peroxisome proliferator-activated receptor α defines a novel positive feedback loop in the rodent liver circadian clock. *Mol. Endocrinol.* 20, 1715–1727. doi: 10.1210/me.2006-0052

Charoensuksai, P., and Xu, W. (2010). PPARs in rhythmic metabolic regulation and implications in health and disease. *PPAR Res.* 2010:2010. doi: 10.1155/2010/243643

Chen, L., and Yang, G. (2014). PPARs integrate the mammalian clock and energy metabolism. *PPAR Res.* 2014:653017. doi: 10.1155/2014/653017

Chen, P., Kakan, X., and Zhang, J. (2010). Altered circadian rhythm of the clock genes in fibrotic livers induced by carbon tetrachloride. *FEBS Lett.* 584, 1597–1601. doi: 10.1016/j.febslet.2010.03.019

Coulon, D., Faure, L., Salmon, M., Wattelet, V., and Bessoule, J.-J. (2012). Occurrence, biosynthesis and functions of N-acylphosphatidylethanolamines (NAPE): not just precursors of N-acylethanolamines (NAE). *Biochimie* 94, 75–85. doi: 10.1016/j.biochi.2011.04.023

Delgado, M. J., Alonso-Gómez, A. L., Gancedo, B., de Pedro, N., Valenciano, A. I., and Alonso-Bedate, M. (1993). Serotonin N-Acetyltransferase (NAT) activity and Melatonin levels in the frog retina are not correlated during the seasonal cycle. *Gen. Comp. Endocrinol.* 92, 143–150. doi: 10.1006/gcen.1993.1151

Diep, T. A., Madsen, A. N., Holst, B., Kristiansen, M. M., Wellner, N., Hansen, S. H., et al. (2011). Dietary fat decreases intestinal levels of the anorectic lipids through a fat sensor. *FASEB J.* 25, 765–774. doi: 10.1096/fj.10-166595

Duggleby, R. G. (1981). A nonlinear regression program for small computers. *Anal. Biochem.* 110, 9–18.

Fu, J., Astarita, G., Gaetani, S., Kim, J., Cravatt, B. F., Mackie, K., et al. (2007). Food intake regulates oleoylethanolamide formation and degradation in the proximal small intestine. *J. Biol. Chem.* 282, 1518–1528. doi: 10.1074/jbc.M607809200

Fu, J., Gaetani, S., Oveisi, F., LoVerme, J., Serrano, A., Rodríguez de Fonseca, F., et al. (2003). Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. *Nature* 425, 90–93. doi: 10.1038/nature01921

Gillum, M. P., Zhang, D., Zhang, X.-M., Erion, D. M., Jamison, R. A., Choi, C., et al. (2008). N-acylphosphatidylethanolamine, a gut-derived circulating factor induced by fat ingestion, inhibits food intake. *Cell* 135, 813–824. doi: 10.1016/j.cell.2008.10.043

Glaser, S. T., and Kaczocha, M. (2009). Temporal changes in mouse brain fatty acid amide hydrolase activity. *Neuroscience* 163, 594–600. doi: 10.1016/j.neuroscience.2009.06.043

Gómez-Boronat, M., Velasco, C., Isorna, E., Pedro, N., Delgado, M. J., and Soengas, J. L. (2016). The satiety factor oleoylethanolamide impacts hepatic lipid and glucose metabolism in goldfish. *J. Comp. Physiol. B* 186, 1009–1021. doi: 10.1007/s00360-016-1009-x

Guijarro, A., Fu, J., Astarita, G., and Piomelli, D. (2010). CD36 gene deletion decreases oleoylethanolamide levels in small intestine of free-feeding mice. *Pharmacol. Res.* 61, 27–33. doi: 10.1016/j.phrs.2009.09.003

Hansen, H. S. (2010). Palmitoylethanolamide and other anandamide congeners. Proposed role in the diseased brain. *Exp. Neurol.* 224, 48–55. doi: 10.1016/j.expneurol.2010.03.022

Hansen, H. S. (2014). Role of anorectic N-acylethanolamines in intestinal physiology and satiety control with respect to dietary fat. *Pharmacol. Res.* 86, 18–25. doi: 10.1016/j.phrs.2014.03.006

Hansen, H. S., and Vana, V. (2018). Non-endocannabinoid N-acylethanolamines and 2-monoacylglycerols in the intestine. *Br. J. Pharmacol.* doi: 10.1111/bph.14175

Hussain, Z., Uyama, T., Tsuboi, K., and Ueda, N. (2017). Mammalian enzymes responsible for the biosynthesis of N-acylethanolamines. *Biochim. Biophys. Acta. Mol. Cell Biol. Lipids* 1862, 1546–1561. doi: 10.1016/j.bbalip.2017.08.006

Inoue, M., Tsuboi, K., Okamoto, Y., Hidaka, M., Uyama, T., Tsutsumi, T., et al. (2017). Peripheral tissue levels and molecular species compositions of N-acyl-phosphatidylethanolamine and its metabolites in mice lacking N-acyl-phosphatidylethanolamine-specific phospholipase D. *J. Biochem.* 162, 449–458. doi: 10.1093/jb/mvx054

Isorna, E., Pedro, N., de Valenciano, A. I., Alonso-Gómez, ÁL., and Delgado, M. J. (2017). Interplay between the endocrine and circadian systems in fishes. *J. Endocrinol.* 232, R141–R159. doi: 10.1530/JOE-16-0330

Izzo, A. A., Piscitelli, F., Capasso, R., Marini, P., Cristino, L., Petrosino, S., et al. (2010). Basal and fasting/refeeding-regulated tissue levels of endogenous PPAR-α ligands in Zucker rats. *Obesity* 18, 55–62. doi: 10.1038/oby.2009.186

Kleberg, K., Hassing, H. A., and Hansen, H. S. (2014). Classical endocannabinoid-like compounds and their regulation by nutrients. *BioFactors* 40, 363–372. doi: 10.1002/biof.1158

Lecarpentier, Y., Claes, V., Duthoit, G., and Hébert, J.-L. (2014). Circadian rhythms, Wnt/beta-catenin pathway and PPAR alpha/gamma profiles in diseases with primary or secondary cardiac dysfunction. *Front. Physiol.* 5:429. doi: 10.3389/fphys.2014.00429

Liedhegner, E. S., Sasman, A., and Hillard, C. J. (2014). Brain region-specific changes in N-acylethanolamine contents with time of day. *J. Neurochem.* 128, 491–506. doi: 10.1111/jnc.12495

Lin, L., Metherel, A. H., Kitson, A. P., Alashmali, S. M., Hopperton, K. E., Trépanier, M.-O., et al. (2018). Dietary fatty acids augment tissue levels of n-acylethanolamines in n-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) knockout mice. *J. Nutr. Biochem.* 62, 134–142. doi: 10.1016/j.jnutbio.2018.08.015

Liu, S., Alexander, R. K., and Lee, C.-H. (2014). Lipid metabolites as metabolic messengers in inter-organ communication. *Trends Endocrinol. Metab.* 25, 356–363. doi: 10.1016/j.tem.2014.05.002

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

LoVerme, J., Fu, J., Astarita, G., Rana, G. L., Russo, R., Calignano, A., et al. (2005). The nuclear receptor peroxisome proliferator-activated receptor-α mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol. Pharmacol.* 67, 15–19. doi: 10.1124/mol.104.006353

Murillo-Rodriguez, E., Désarnaud, F., and Prospéro-García, O. (2006). Diurnal variation of arachidonoylethanolamine, palmitoylethanolamide and oleoylethanolamide in the brain of the rat. *Life Sci.* 79, 30–37. doi: 10.1016/j.lfs.2005.12.028

Nisembaum, L. G., Velarde, E., Tinoco, A. B., Azpeleta, C., de Pedro, N., Alonso-Gómez, A. L., et al. (2012). Light-dark cycle and feeding time differentially entrains the gut molecular clock of the goldfish (*Carassius auratus*). *Chronobiol. Int.* 29, 665–673. doi: 10.3109/07420528.2012.686947

Paredes, J. F., López-Olmeda, J. F., Martínez, F. J., and Sánchez-Vázquez, F. J. (2015). Daily rhythms of lipid metabolic gene expression in zebra fish liver: response to light/dark and feeding cycles. *Chronobiol. Int.* 32, 1438–1448. doi: 10.3109/07420528.2015.1104327

Paredes, J. F., Vera, L. M., Martinez-Lopez, F. J., Navarro, I., and Vázquez, F. J. S. (2014). Circadian rhythms of gene expression of lipid metabolism in Gilthead sea bream liver: synchronisation to light and feeding time. *Chronobiol. Int.* 31, 613–626. doi: 10.3109/07420528.2014.881837

Petersen, G., Sørensen, C., Schmid, P. C., Artmann, A., Tang-Christensen, M., Hansen, S. H., et al. (2006). Intestinal levels of anandamide and oleoylethanolamide in food-deprived rats are regulated through their precursors. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* 1761, 143–150. doi: 10.1016/j.bbalip.2005.12.011

Piomelli, D. (2013). A fatty gut feeling. *Trends Endocrinol. Metab.* 24, 332–341. doi: 10.1016/j.tem.2013.03.001

Rahman, I. A. S., Tsuboi, K., Uyama, T., and Ueda, N. (2014). New players in the fatty acyl ethanolamide metabolism. *Pharmacol. Res.* 86, 1–10. doi: 10.1016/j.phrs.2014.04.001

Ribas-Latre, A., and Eckel-Mahan, K. (2016). Interdependence of nutrient metabolism and the circadian clock system: importance for metabolic health. *Mol. Metab.* 5, 133–152. doi: 10.1016/j.molmet.2015.12.006

Rodríguez de Fonseca, F., Navarro, M., Gómez, R., Escuredo, L., Nava, F., Fu, J., et al. (2001). An anorexic lipid mediator regulated by feeding. *Nature* 414, 209–212. doi: 10.1038/35102582

Romano, A., Tempesta, B., Provensi, G., Passani, M. B., and Gaetani, S. (2015). Central mechanisms mediating the hypophagic effects of oleoylethanolamide and N-acylphosphatidylethanolamines: different lipid signals? *Front. Pharmacol.* 6:137. doi: 10.3389/fphar.2015.00137

Shimba, S., Ishii, N., Ohta, Y., Ohno, T., Watabe, Y., Hayashi, M., et al. (2005). Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12071–12076. doi: 10.1073/pnas.0502383102

Shimba, S., Ogawa, T., Hitosugi, S., Ichihashi, Y., Nakadaira, Y., Kobayashi, M., et al. (2011). Deficient of a clock gene, brain and muscle arnt-like protein-1 (BMAL1), induces dyslipidemia and ectopic fat formation. *PLoS One* 6:e25231. doi: 10.1371/journal.pone.0025231

Sihag, J., and Jones, P. J. H. (2018). Oleoylethanolamide: the role of a bioactive lipid amide in modulating eating behaviour. *Obes. Rev.* 19, 178–197. doi: 10.1111/obr.12630

Terrazzino, S., Berto, F., Carbonare, M. D., Fabris, M., Guiotto, A., Bernardini, D., et al. (2004). Stearoylethanolamide exerts anorexic effects in mice via down-regulation of liver stearoyl-coenzyme A desaturase-1 mRNA expression. *FASEB J.* 18, 1580–1582. doi: 10.1096/fj.03-1080fje

Tinoco, A. B., Armirotti, A., Isorna, E., Delgado, M. J., Piomelli, D., and de Pedro, N. (2014). Role of oleoylethanolamide as a feeding regulator in goldfish. *J. Exp. Biol.* 217, 2761–2769. doi: 10.1242/jeb.106161

Tsuboi, K., Ikematsu, N., Uyama, T., Deutsch, D. G., Tokumura, A., and Ueda, N. (2013). Biosynthetic pathways of bioactive N-acylethanolamines in brain. *CNS Neurol. Disord. Drug Targets* 12, 7–16. doi: 10.2174/1871527311312010005

Ueda, N., Tsuboi, K., and Uyama, T. (2013). Metabolism of endocannabinoids and related N-acylethanolamines: canonical and alternative pathways. *FEBS J.* 280, 1874–1894. doi: 10.1111/febs.12152

Valenti, M., Viganò, D., Casico, M. G., Rubino, T., Steardo, L., Parolaro, D., et al. (2004). Differential diurnal variations of anandamide and 2-arachidonoylglycerol levels in rat brain. *Cell. Mol. Life Sci.* 61, 945–950. doi: 10.1007/s00018-003-3453-5

Wang, Q., Tikhonenko, M., Bozack, S. N., Lydic, T. A., Yan, L., Panchy, N. L., et al. (2014). Changes in the daily rhythm of lipid metabolism in the diabetic retina. *PLoS One* 9:e95028. doi: 10.1371/journal.pone.0095028

Wellner, N., Tsuboi, K., Madsen, A. N., Holst, B., Diep, T. A., Nakao, M., et al. (2011). Studies on the anorectic effect of N-acylphosphatidylethanolamine and phosphatidylethanolamine in mice. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* 1811, 508–512. doi: 10.1016/j.bbalip.2011.06.020

Yang, X., Downes, M., Yu, R. T., Bookout, A. L., He, W., Straume, M., et al. (2006). Nuclear receptor expression links the circadian clock to metabolism. *Cell* 126, 801–810. doi: 10.1016/j.cell.2006.06.050

Zhang, D., Tong, X., Arthurs, B., Guha, A., Rui, L., Kamath, A., et al. (2014). Liver clock protein BMAL1 promotes de novo lipogenesis through insulin-mTORC2-AKT signaling. *J. Biol. Chem.* 289, 25925–25935. doi: 10.1074/jbc.M114.567628

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer NL declared a shared affiliation, with no collaboration, with one of the authors, DP, to the handling Editor at the time of review.

Supplementary Online Material

Diurnal profiles of N-acylethanolamines in goldfish brain and gastrointestinal tract: possible role of feeding

Miguel Gómez-Boronat[1], Esther Isorna[1], Andrea Armirotti[2], María J. Delgado[1], Daniele Piomelli[3] and Nuria de Pedro[1]

Table S1. Complete panel of source parameters and MRM transitions of NAEs.

NAEs – MRM Transitions	Abbreviation used in the text	Retention Time (min)	Parent ion (m/z)	Daughter ion (m/z)	Collision Energy (V)
Oleoylethanolamide (d18:1)	OEA	2.70	326	62	20
Oleoylethanolamide (d18:1)	OEA–d_4	2.70	330	66	20
Palmitoylethanolamide (d16:0)	PEA	2.65	300	62	20
Palmitoylethanolamide (d16:0)	PEA–d_4	2.65	304	66	20
Stearoylethanolamide (d18:0)	SEA	2.90	328	62	20
Stearoylethanolamide (d18:0)	SEA–d_3	2.89	331	66	20

Table S2. Complete panel of source parameters and MRM transitions of NAPEs.

NAPEs – MRM Transitions	Abbreviation used in the text	Retention Time (min)	Parent ion (m/z)	Daughter ion (m/z)	Collision Energy (V)
16:0-18:3-N18:1 NAPE	NAPE OEA	5.38	994.7	308.3	20
16:0-20:4-N18:1 NAPE		5.17	1020.7	308.3	20
16:0-22:5-N18:1 NAPE		4.92	1046.7	308.3	20
16:0-22:6-N18:1 NAPE		4.93	1044.7	308.3	20
18:0-18:1-N18:1 NAPE		5.09	1026.8	308.3	20
18:0-18:3-N18:1 NAPE		4.98	1022.7	308.3	20
18:0-20:4-N18:1 NAPE		5.03	1048.7	308.3	20
18:0-22:5-N18:1 NAPE		5.09	1074.8	308.3	20
18:0-22:6-N18:1 NAPE		4.94	1072.7	308.3	20
18:1-18:1-N18:1 NAPE		5.10	1024.7	308.3	20
18:1-18:2-N18:1 NAPE		5.50	1022.7	308.3	20
18:1-18:3-N18:1 NAPE		5.39	1020.7	308.3	20
18:1-20:4-N18:1 NAPE		5.20	1046.7	308.3	20
18:1-22:5-N18:1 NAPE		5.10	1072.7	308.3	20
18:1-22:6-N18:1 NAPE		5.16	1070.7	308.3	20
16:0-16:0-N16:0 NAPE	NAPE PEA	3.53	946.7	224.2	20
16:0-18:1-N16:0 NAPE		3.53	972.7	224.2	20
18:0-22:6-N16:0 NAPE		3.49	1046.7	224.2	20
P18:0-22:6-N16:0 NAPE		3.56	1077.7	224.2	20

Table S2 (continue). Complete panel of source parameters and MRM transitions of NAPEs.

16:0-18:1-N18:0 NAPE	NAPE SEA	3.53	1000.8	252.3	20
18:0-20:4-N18:0 NAPE		3.63	1050.8	252.3	20
16:0-22:6-N18:0 NAPE		3.49	1046.7	252.3	20
P16:0-22:6-N18:0 NAPE		3.56	1077.7	252.3	20
18:0-22:6-N18:0 NAPE		3.60	1074.8	252.3	20
P18:0-22:6-N18:0 NAPE		3.66	1105.7	252.3	20
16:0-16:0-N17:0 NAPE	IS NAPE	3.55	960.7	238.3	20
18:0-22:6-N17:0 NAPE		5.14	1056.7	238.3	20

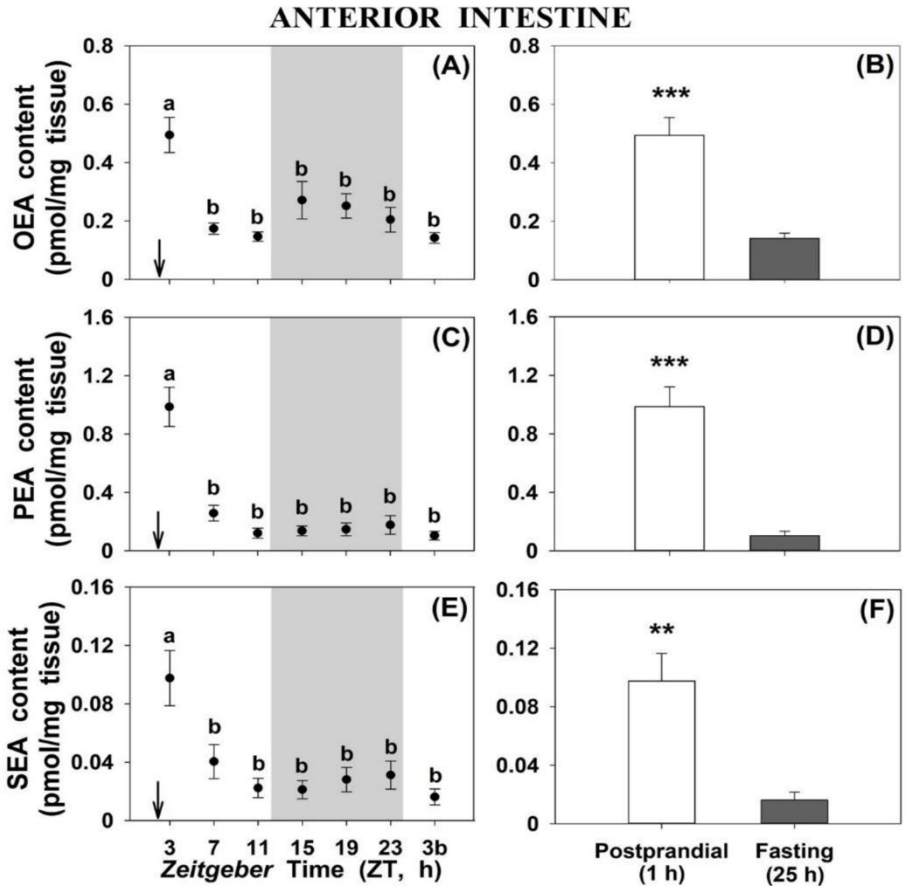


Figure S1. Daily variations of NAEs (OEA, PEA, and SEA) content in anterior intestine of goldfish. **A**, **C**, and **E**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). *Different letters* indicate significant differences among groups (ANOVA, $p < 0.05$ and SNK post-hoc test). **B**, **D**, and **F**, comparison between 1 h postprandial, ZT3, and 25 h fasting, ZT3b. *Asterisks* indicate significant differences by Student *t-test* analysis (** $p < 0.01$, *** $p < 0.001$). Data are shown as mean ± SEM (n = 6-7).

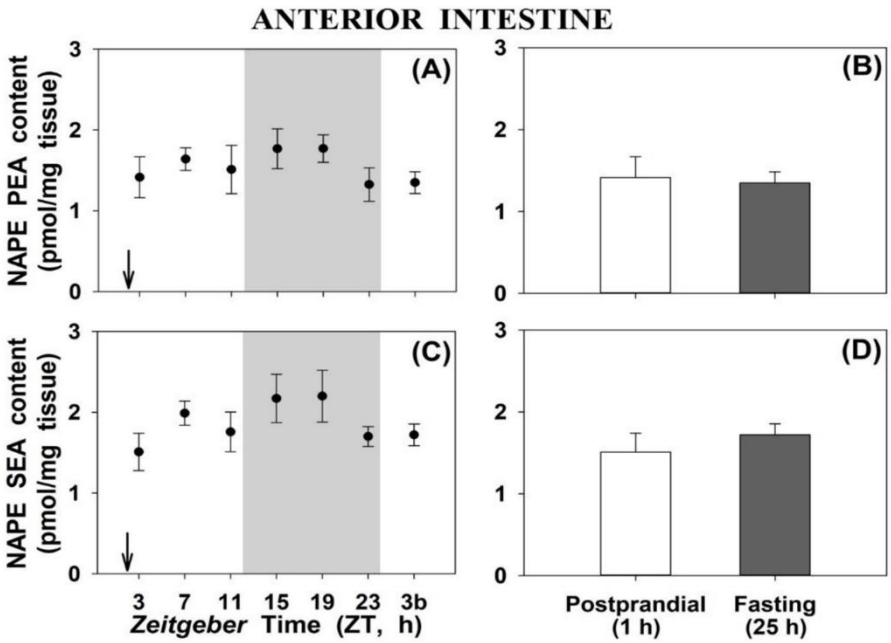


Figure S2. Daily variations of NAPEs content in anterior intestine of goldfish. **A**, **C**, and **E**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). **B**, **D**, and **F**, comparison between 1 h postprandial, ZT3, and 25 h fasting, ZT3b. Data are shown as mean ± SEM (n = 6-7).

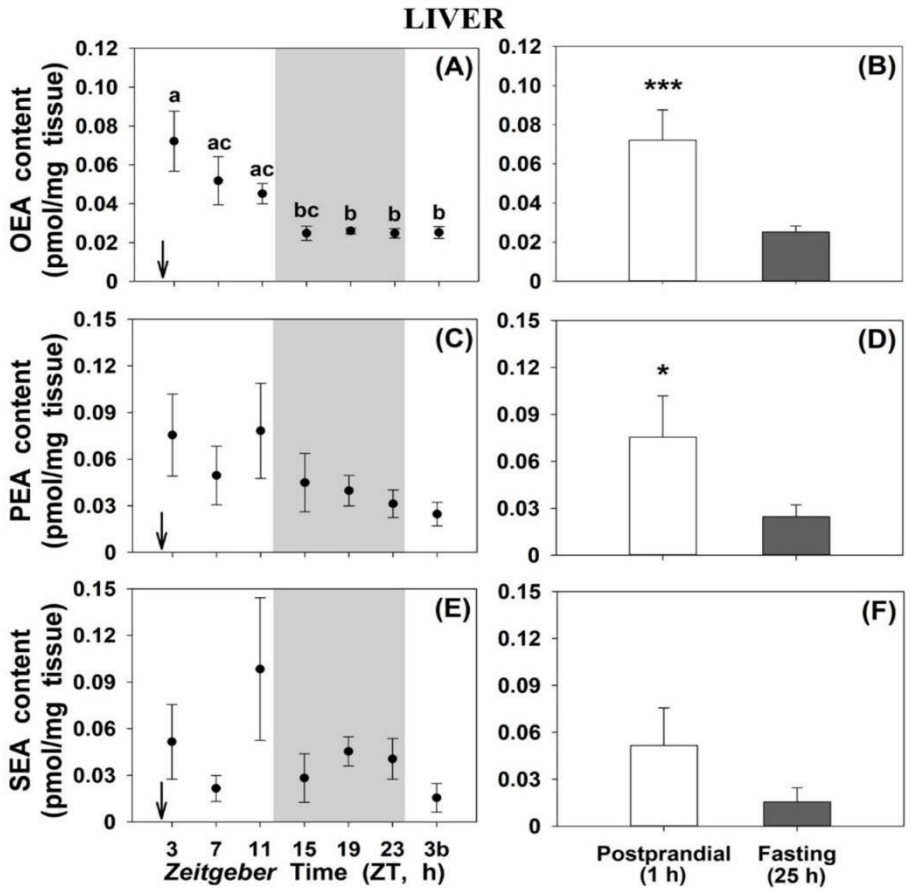


Figure S3. Daily variations of NAEs (OEA, PEA, and SEA) content in liver of goldfish. **A**, **C**, and **E**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). *Different letters* indicate significant differences among groups (ANOVA, $p < 0.05$ and SNK post-hoc test). **B**, **D**, and **F**, comparison between 1 h postprandial, ZT3, and 25 h fasting, ZT3b. *Asterisks* indicate significant differences by Student *t-test* analysis (* $p < 0.05$, *** $p < 0.001$). Data are shown as mean ± SEM (n = 6-7).

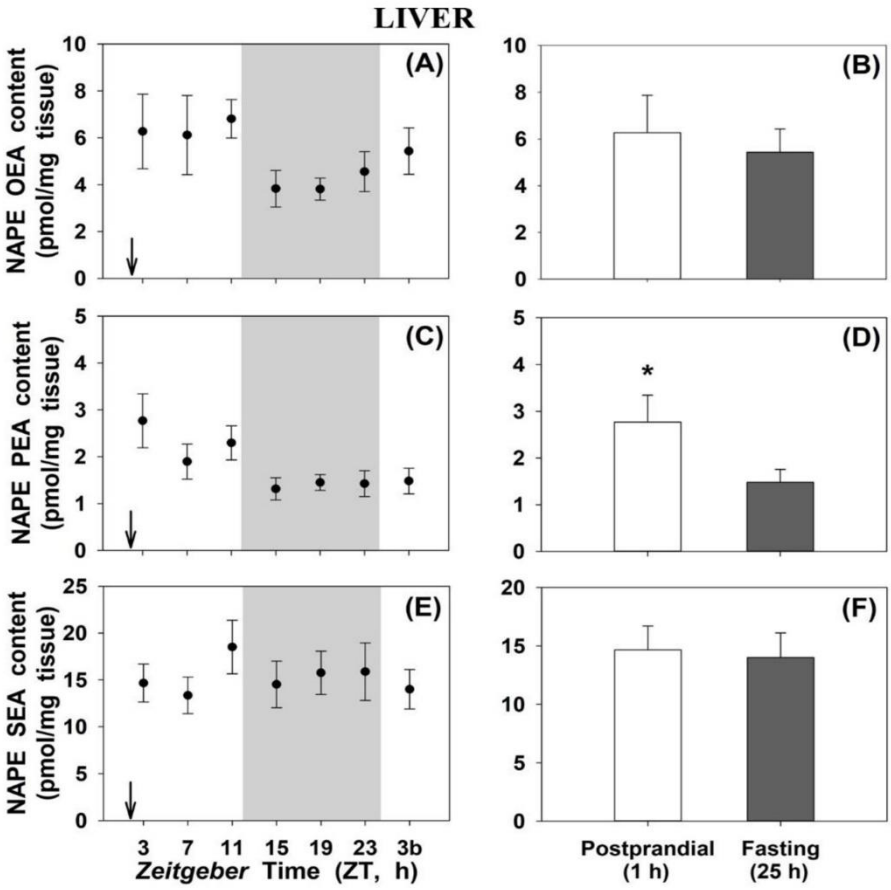


Figure S4. Daily variations of NAPEs content in liver of goldfish. **A**, **C**, and **E**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). **B**, **D**, and **F**, comparison between 1 h postprandial, ZT3, and 25 h fasting, ZT3b. *Asterisks* indicate significant differences by Student *t-test* analysis (* $p < 0.05$). Data are shown as mean ± SEM (n = 6-7).

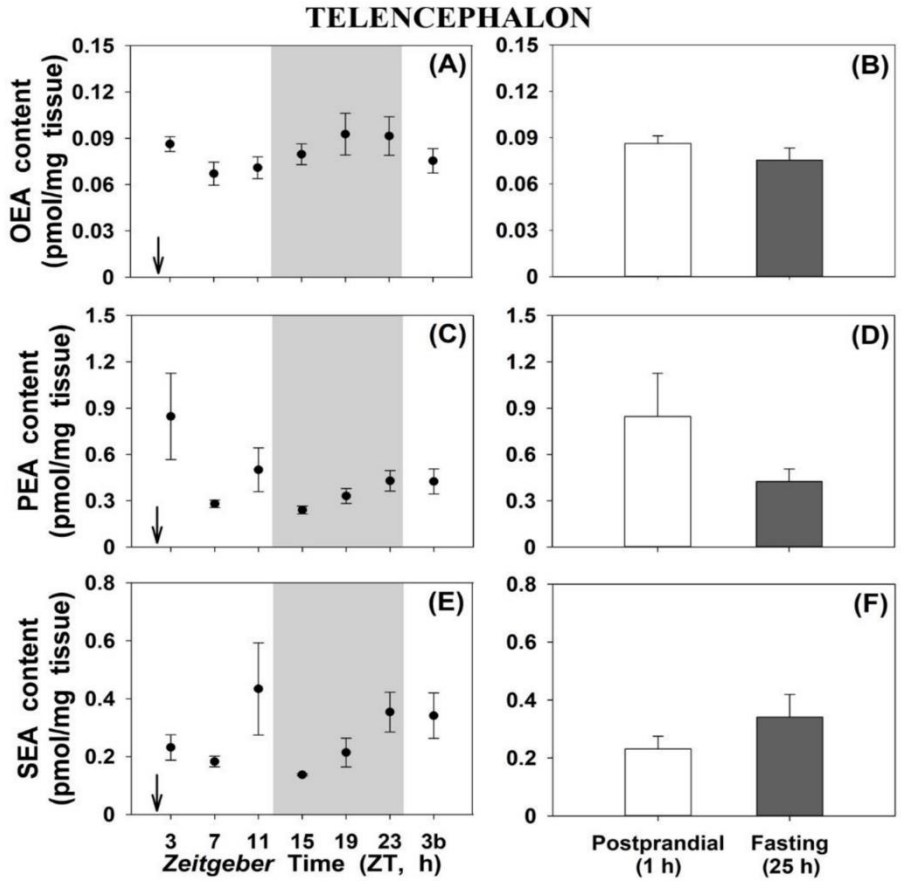


Figure S5. Daily variations of NAEs (OEA, PEA, and SEA) content in telencephalon of goldfish. **A**, **C**, and **E**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). **B**, **D**, and **F**, comparison between 1 h postprandial, ZT3, and 25 h fasting, ZT3b. Data are shown as mean ± SEM (n = 6-7).

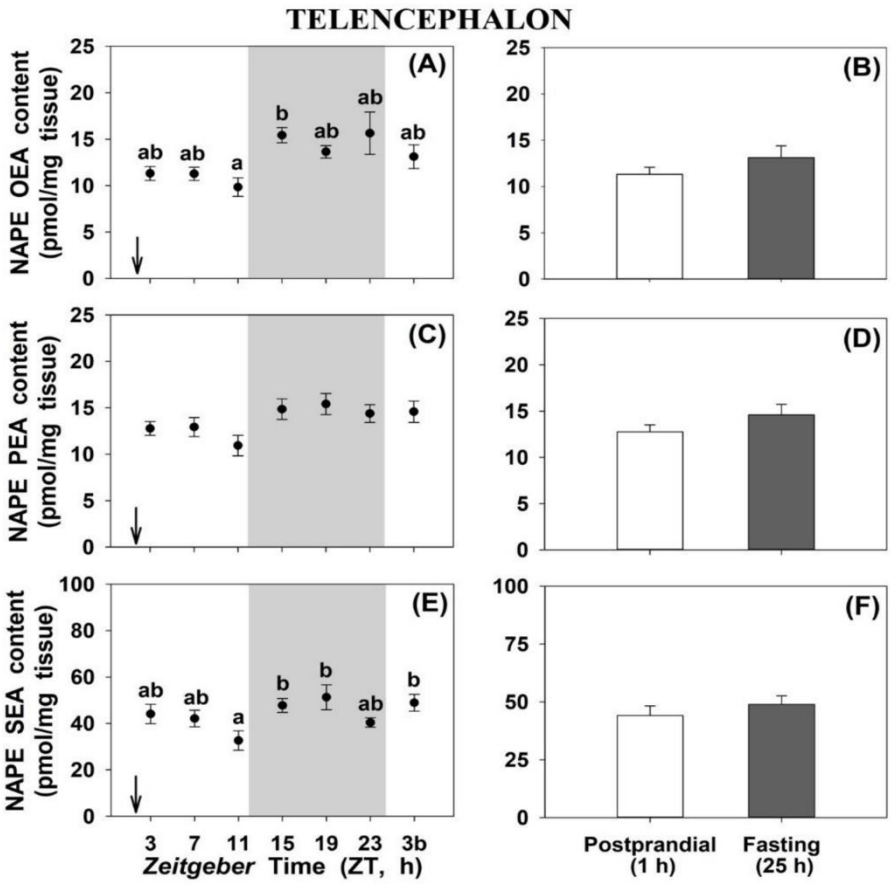


Figure S6. Daily variations of NAPEs content in telencephalon of goldfish. **A**, **C**, and **E**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). *Different letters* indicate significant differences among groups (ANOVA, $p < 0.05$ and SNK post-hoc test). **B**, **D**, and **F**, comparison between 1 h postprandial, ZT3, and 25 h fasting, ZT3b. Data are shown as mean ± SEM (n = 6-7).

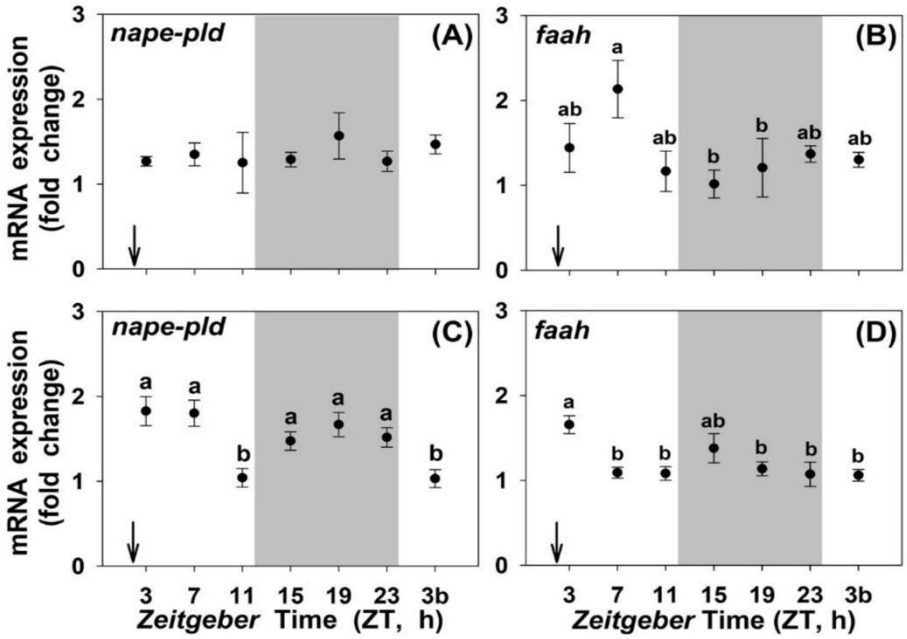


Figure S7. Daily rhythms of *nape-pld* **(A** and **C)** and *faah* **(B** and **D)** expression in anterior intestine **(A** and **B)** and liver **(C** and **D)** of goldfish. Gray area indicates the dark phase and arrows the feeding time (ZT2). Data are shown as mean ± SEM (n = 6-7) in relative units ($2^{-\Delta\Delta Ct}$ method). *Different letters* indicate significant differences among groups (ANOVA, $p < 0.05$ and SNK post-hoc test).

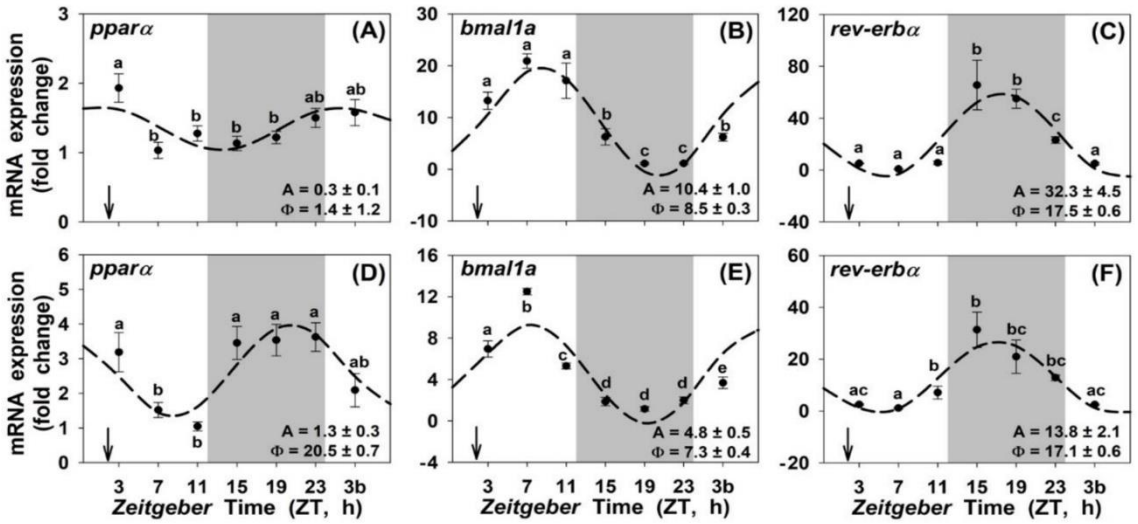


Figure S8. Daily rhythms of *pparα* **(A** and **D)**, *bmal1a* **(B** and **E)** and *rev-erbα* **(C** and **F)** expression in anterior intestine **(A-C)** and liver **(D-F)** of goldfish. Gray area indicates the dark phase and arrows the feeding time (ZT2). Data are shown as mean ± SEM (n = 6-7) in relative units ($2^{-\Delta\Delta Ct}$ method). *Different letters* indicate significant differences among groups (ANOVA, $p < 0.05$, and SNK post-hoc test). Dashed lines represent periodic sinusoidal functions when rhythms are significant (Cosinor, $SE(A)/A < 0.3$; and ANOVA, $p < 0.05$). Amplitude (A) and acrophase (Φ) values of the rhythms are shown in the top-right of the graphs.

CAPÍTULO 2

Sistema circadiano y homeostasis temporal

CAPÍTULO 2

Sistema circadiano y homeostasis temporal

2.1 Ritmos diarios de receptores nucleares y genes reloj en el hipotálamo y el hígado del carpín bajo diferentes condiciones de luminosidad.

Nuclear receptors y clock genes rhythms in hypothalamus and liver of goldfish under different lighting conditions.

***Chronobiology International* (2019).**

En revisión

2.2 Un desfase en el horario de la alimentación actúa como un agente estresante que altera los osciladores circadianos en el carpín.

Time-lag in feeding schedule acts as a stressor that alters circadian osicllators in goldfish.

***Frontiers in Physiology* (2018) 9:1749.**

doi: 10.3389/fphys.2018.01749

2.3 Influencia del horario de alimentación sobre los receptores nucleares PPARα y REV-ERBα en el hipotálamo y el hígado de carpín.

Los principales resultados obtenidos en los tres trabajos que configuran el Capítulo 2 de la presente Tesis Doctoral se recogen en la **Figura 17**.

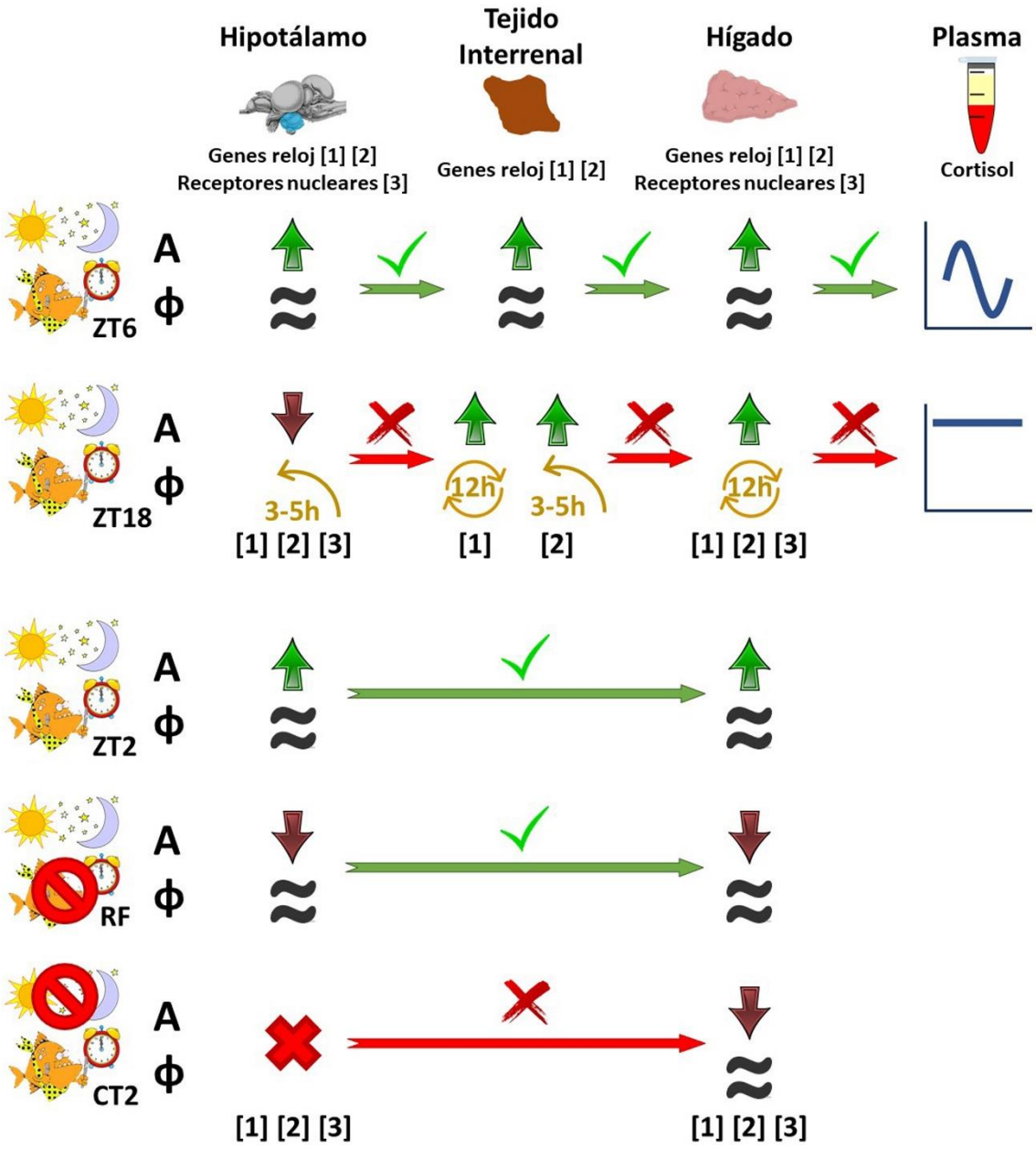
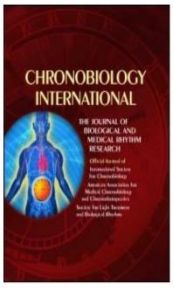


Figura 17. Resumen de los resultados del Capítulo 2. [1] genes *per*; [2] genes *clock1a*/*bmal1a*; [3] receptores nucleares (*rev-erbα*, *rev-erbβ*, *pparα*, *pparγ* y *rorα*). A, amplitud; ϕ, acrofase; CT, tiempo circadiano; RF, alimentación aleatoria; ZT, tiempo *zeitgeber*. ✓, sincronización; ✗, desincronización; ✖, no ritmos.



Taylor & Francis
Taylor & Francis Group

Chronobiology International

The Journal of Biological and Medical Rhythm Research

ISSN: 0742-0528 (Print) 1525-6073 (Online) Journal homepage: http://www.tandfonline.com/loi/icbi20

Nuclear receptors and clock genes rhythms in hypothalamus and liver of goldfish under different lighting conditions

Miguel Gómez-Boronat, Nuria de Pedro, María J. Delgado & Esther Isorna

To cite this article: Miguel Gómez-Boronat, Nuria de Pedro, María J. Delgado & Esther Isorna (2019): Nuclear receptors and clock genes rhythms in hypothalamus and liver of goldfish under different lighting conditions, Chronobiology International, UNDER REVIEW.

To link to this article: –

CHRONOBIOLOGY INTERNATIONAL
Under review

Taylor & Francis
Taylor & Francis Group

Check for updates

Nuclear Receptors and Clock Genes Rhythms in Hypothalamus and Liver of Goldfish under Different Lighting Conditions

Miguel Gómez-Boronat, Nuria de Pedro, María J. Delgado, and Esther Isorna

Departamento de Genética, Fisiología y Microbiología, Unidad Docente de Fisiología Animal, Facultad de Biología, Universidad Complutense de Madrid, Spain.

ABSTRACT

The circadian system is an intricated network of oscillators placed in central and peripheral tissues that are tightly linked to produce circadian endogenous rhythms in all vertebrates, in order to adapt the organism to the cyclic environmental changes. Nuclear receptors – such as PPARs, REV-ERBs, and RORs – are a superfamily of ligand-activated transcription factors that confer greater robustness to the circadian system modulating the clock-genes transcription of the core clock. In addition, these nuclear receptors regulate a large number of metabolic processes related to glucose and lipid metabolism, among others. Due to these two key qualities of nuclear receptors, PPARs, REV-ERBs, and RORs have been proposed as the main genes that exert the link between metabolism and circadian system. To date, it is unclear whether these nuclear receptors are rhythmic or whether the light-dark and fasting-feeding cycles are important for their synchronization in fish. Therefore, the objective of this study was to investigate whether the two main *zeitgebers* (light-dark cycle and feeding time) could affect the synchronization of central (hypothalamus) and peripheral (liver) core clocks and of the nuclear receptors in goldfish (*Carassius auratus*). To this aim, three experimental groups were established: fish under a 12 h light-12 h darkness with scheduled feeding at ZT2, fish with the same photoperiod but randomly fed, and fish under constant darkness (24D) and scheduled feeding at CT2. After one month, clock genes and nuclear receptors expression in hypothalamus and liver, and circulating glucose were studied. Results showed that clock genes displayed daily rhythms in hypothalamus as well as in liver of goldfish under the presence of both *zeitgebers*, with the acrophases of *clock1a* and *bmal1a* in antiphase with that of *per1* genes, as expected for proper functioning clocks. In random-fed fish both oscillators remained unchanged, but in 24D-fish hypothalamic clock genes were fully arrhythmic while the hepatic ones were still rhythmic. Regarding the nuclear receptors, we can observe that in the hypothalamus only *rev-erba* was rhythmic under a light-dark alternating photoperiod, while in the liver all nuclear receptors were rhythmic when both *zeitgebers* were present but only *rev-erba* when one of them were removed. Plasma glucose levels showed significant rhythms in fishes under a random fed regimen or a constant darkness photoperiod with the highest levels in all groups 1-h postprandial. Altogether, results indicate that hypothalamus in mainly controlled by the light-dark cycle and the liver by the feeding-fasting cycle. Moreover, nuclear receptors are being discovered as authentic outputs of the circadian system acting as very important molecules for the maintenance of temporal homeostasis, independently of the "energy state" in fish.

ARTICLE HISTORY

KEYWORDS:
Fish; circadian system; REV-ERB; PPAR; ROR; clock genes; light-dark cycle

Introduction

Nuclear receptors (NRs) are a superfamily of ligand-activated transcription factors that regulate gene transcription acting on essential physiological processes such as growth, development, immunity, and metabolic homeostasis (Eckel-Mahan and Sassone-Corsi, 2013; Kojetin and Burris, 2014; Ribas-Latre and Eckel-Mahan, 2016). All NRs described share a great homology in their amino acid sequence with highly conserved domain structures (Solt et al., 2011). Some examples of classical and well-known NRs include the estrogen receptor, progesterone receptor, glucocorticoid receptor and thyroid receptor. However, recent studies have advanced in the knowledge of three subfamilies of NRs participating as critical regulators of circadian clock with significant roles in lipid homeostasis (Solt et al., 2011). These three subfamilies are the nuclear receptor subfamily 1 group C (NR1C) or commonly named as PPAR, the nuclear receptor subfamily 1 group D (NR1D) or REV-ERB, and the nuclear receptor subfamily 1 group F (NR1F) or retinoic acid-related orphan receptor (ROR) (Nuclear Receptors Nomenclature Committee, 1999; Weikum et al., 2018).

As regulators of genes involved in lipid and glucose metabolism, lipid storage and lipogenesis, hepatic fatty acid oxidation, or ketogenesis among

CONTACT Esther Isorna eisornaa@bio.ucm.es Departamento de Genética, Fisiología y Microbiología, Unidad Docente de Fisiología Animal, Facultad de Biología, Universidad Complutense de Madrid, 28039, Madrid, Spain

Supplementary Online Material

Nuclear Receptors and Clock Genes Rhythms in Hypothalamus and Liver of Goldfish under Different Lighting Conditions

Miguel Gómez-Boronat, Nuria de Pedro, María J. Delgado, Esther Isorna*

Methods

Partial cDNA sequencing of goldfish rev-erbβ b and rorα a and phylogenetic analysis

To obtain a partial sequence of goldfish *rev-erbβ b* and *rorα a*, total RNA from hypothalamus and liver was isolated using TRI® Reagent (Sigma-Aldrich, Madrid, Spain) and treated with RQ1 RNase-Free DNase (Promega, Madison, USA) according to the manufacturer's instructions. Then, an aliquot of 1 µg of total RNA was reverse transcribed into cDNA in a 25 µl reaction volume using random primers (Invitrogen, Carlsbad, USA), RNase inhibitor (Promega), and SuperScript II Reverse Transcriptase (Invitrogen). The reverse transcription reaction conditions consisted in 25 ºC for 10 min, an extension of 50 min at 42 ºC, and a denaturalization step at 70 ºC for 15 min.

The first strand cDNA fragments obtained were used as a template to amplify *rev-erbβ b* and *rorαa* using various sets of primers (**Table S1**), all obtained from Sigma-Aldrich. Primers were hand-made designed by selecting convergent parts between the transcription factors' nucleotide sequence of *Danio rerio* (GenBank IDs: *rev-erbβ b*: NM_131065.1; *rorα a*: NM_001110167.1), *Cyprinus carpio* (GenBank IDs: *rev-erbβ b*: XM_019124111.1; *rorα a*: XM_019100303.1), and *Sinocyclocheilus grahami* (GenBank IDs: *rev-erbβ b*: XM_016256081.1; *rorα a*: XM_016290300.1) using the Clustal Omega alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). The PCRs were performed in a 25-µl reaction volume containing 1.25 U of TaqDNA Polymerase recombinant, PCR Buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM of $MgCl_2$, 0.2 mM of dNTP mixture (all from Invitrogen), 0.2 µM of each forward and reverse primer, and 1 µl of cDNA. Reactions were performed in a 50-µl final volume containing 1.25 U of GoTaq Hot Start Polymerase, PCR Buffer, 2 mM of $MgCl_2$ (all from Promega), 0.2 mM of dNTP mixture (Invitrogen), 0.2 µM of each forward and reverse primer, and 2 µl of cDNA. Cycling conditions comprised an initial incubation at 94 ºC for 3 min, followed by 40 cycles of 94 ºC for 45 sec, 60 ºC for 30 sec, and 72 ºC for 60 sec, with a final extension step at 72 ºC for 5 min. The PCR products were electrophoresed on a 1.5 % agarose gel to check the target bands from each PCR and, in all those that matched the expected amplicons, the PCR products were then purified using UltraClean® PCR Clean-Up Kit (Mo Bio Laboratories Inc., Carlsbad, USA) and sequenced (Secugen, Madrid, Spain). The nucleotide deduced sequences were analyzed with the BLAST program on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/).

Table S1. Primers designed for identifying the cDNA sequences of *rev-erbβ* and *rorα a* in goldfish.

Gene	Primer Name	Sequence (5' → 3')
rev-erbβ b	Forward 1	GAGAAGCTGCAAGCTCTGAAC
	Forward 2	CGCAGCTTGATCACCAAGAA
	Reverse 1	AGAGTTTCCTGAAGGGCCTC
	Reverse 2	AAGAGCTGCTCAGAGTGCA
rorα a	Forward 1	GGACCGCCAAGCTTAATGATGTA
	Forward 2	AAGTCATGTGGCAGTTGTGTG
	Reverse 1	AAGCGCTACCCATCAACGG
	Reverse 2	CTGATCATTCTGACAGAGCTCCA

rev-erb, nuclear receptor subfamily 1 group D (nr1d); *rorα a*, retinoic acid-related orphan receptor alpha (A) paralog a.

Phylogenetic analysis was performed by aligning the goldfish *rev-erbβ b* and *rorα a* sequences with those of other vertebrates retrieved from GenBank (NCBI) using the Clustal Omega alignment tool. Alignment was performed using the partial fragment of nucleotide sequences for each species, which coincide with the obtained sequence for goldfish. A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987), with 1000 replicates for the bootstrap test, using MEGA6 (Tamura et al., 2013). The *Lepisosteus oculatus* was considered as outgroup in the phylogenetic analysis given the basal phylogenetic position of chondrichthyan fish.

Results

Two partial fragments of 248 bp and 1381 bp in length were sequenced from goldfish liver cDNA, which respectively correspond to *rev-erbβ b* (GenBank accession no. MH674345) and *rorα a* (GenBank accession no. MK033601). The alignment of the deduced amino acid sequences from these cDNA fragments and the corresponding fragments from *D. rerio*, *C. carpio*, and *S. grahami* sequences are shown in Fig. S1. Being consider the sequence of *D. rerio* as the reference for the beginning, the partial peptide sequence obtained for goldfish REV-ERBβ B corresponds with the amino acid positions Glu489-Leu571 of *D. rerio* sequence, Glu397-Leu479 of *C. carpio*, and Glu468-Leu550 of *S. grahami*, while the partial peptide sequence obtained for goldfish RORα

Asp467 of *D. rerio* sequence, Met93-Asp467 of *C. carpio*, and Ala8-Asp467 of *S. grahami*. The alignment shows a high degree of identity between the obtained goldfish sequences and those from the other three teleosts in both genes.

The phylogenetic analysis comparing the goldfish *rev-erbβ* and *rorα* nucleotide sequences and those from other vertebrates (Fig. S2) positions both respective sequences of *L. oculatus* in the base of the evolutionary scale, as expected. In Teleostei, whose ancestor underwent a whole genome duplication (3R), both genes are duplicated, and the paralogs are named *rev-erbβ/rorα a* and *rev-erbβ/rorα b*. Note that, during the bioinformatic analysis here performed, two different genes (named 1 and 2) were detected for the four paralogs (*rev-erbβ a*, *rev-erbβ b*, *rorα a*, and *rorα b*) in members of the Cyprininae subfamily (*C. auratus*, *C. carpio*, *S. grahami*, *S. anshuiensis*, and *S. rhinocerous*), probably reflecting the whole genome duplication in Cyprininae (4R). As the obtained sequences are part of a highly conserved region in both cases (Fig. S1), the phylogenetic analysis allowed us to correctly classify them into the paralogs *rev-erbβ b* and *rorα a*, and more deeply in the second gene, we can assume that we obtained the paralog *rorα a 1*. In the case of REV-ERBβ b, we cannot confirm which paralog we have obtained since, as it can be seen in the phylogenetic tree (Fig. S2), there is no perfect separation between *rev-erbβ b 1* and *rev-erbβ b 2*. Furthermore, the phylogenetic analysis also allowed us to correct the classification of some paralogs due to the similarity between the different orthologs. In this way, the sequence of *C. carpio* named as *rev-erbβ a* should be renamed as *rev-erbβ b*. For its part, within the paralog *rorα a*, the sequence of *C. auratus* placed in the paralog *rorα a 2* should be renamed in the same way, while the sequences of *S. anshuiensis* should be swapped between paralogs 1 and 2. Finally, within the paralog *rorα b*, the sequence of *S. anshuiensis* placed in the paralog *rorα b 1* should be renamed in the same way.

Rev-erbβ b

```
C. auratus          EKLQALNLSEEEMSLFTAVVLVSADRSGLENVNSVEALQETLIRALRSLITKNHPNEIAI
D. rerio      489   EKLQALNLSEEEMSLFTAVVLVSADRSGLENVNSVEALQETLIRALRSLITKNHPNEIAI  548
C. carpio     397   EKLQALNLSEEEMSLFTAVVLVSADRSGLENVNSVEALQETLIRALRSLITKNHPNEIAI  456
S. grahami    468   EKLQALNLSEEEMSLFTAVVLVSADRSGLENVNSVEALQETLIRALRSLITKNHPNELAI  527
                    ************************************************************:**

C. auratus          FTKLLLKLPDLRSLNNMHSEQL
D. rerio      549   FTKLLLKLPDLRSLNNMHSEQL  571
C. carpio     457   FTKLLLKLPDLRSLNNMHSEQL  479
S. grahami    528   FTKLLLKLPDLRSLNNMHSEQL  550
                    **********************
```

Rorα a

```
C. auratus          AMKAQIEIIPCKICGDKSSGIHYGVITCEGCKGFFRRSQQSNAAYSCPRQKNCLIDRTSR
C. carpio     0     ------------------------------------------------------------  0
S. grahami    8     AMKAQIEIIPCKICGDKSSGIHYGVITCEGCKGFFRRSQQSNAAYSCPRQKNCLIDRTSR  67
D. rerio      8     AMKAQIEIIPCKICGDKSSGIHYGVITCEGCKGFFRRSQQSNAAYSCPRQKNCLIDRTSR  67
                    ************************************************************

C. auratus          NRCQHCRLQKCLAVGMSRDAVKFGRMSKKQRDSLYAEVQKHRLQQQQRDHQQQPGEAEPL
C. carpio     0     -----------------------MSKKQRDSLYAEVQKHRLQQQQRDHQQQPGEAEPL  35
S. grahami    68    NRCQHCRLQKCLAVGMSRDAVKFGRMSKKQRDSLYAEVQKHRLQQQQREHQQQPGEAEPL  127
D. rerio      68    NRCQHCRLQKCLAVGMSRDAVKFGRMSKKQRDSLYAEVQKHRLQQQQRDHQQQPGEAEPL  127
                    ************************************************:***********

C. auratus          TPTYGLSTNGLTELHDDLSGYMNGHTPDGTKPDSGVSSFYLDIQPSPDQSGLDINGIKPE
C. carpio     36    TPTYGLSTNGLTELHDDLSGYMNGHTPDGTKPDSGVSSFYLDIQPSPDQSGLDINGIKPE  95
S. grahami    129   TPTYGLSTNGLTELHDDLSGYMNGHTPDGTKPDSGVSSFYLDIQPSPDQSGLDINGIKPE  187
D. rerio      129   TPTYGLSTNGLTELHDDLSGYMNGHTPDGTKPDSGVSSFYLDIQPSPDQSGLDINGIKPE  187
                    ************************************************************

C. auratus          PICDFTPGSGFFPYCSFTNGETSPTVSMAELEHLAQNISKSHMETCQYLREELQQMTWQA
C. carpio     96    PICDFTPGSGFFPYCSFTNGETSPTVSMAELEHLAQNISKSHMETCQYLREELQQMTWQA  155
S. grahami    189   PICDFTPGSGFFPYCSFTNGETSPTVSMAELEHLAQNISKSHMETCQYLREELQQMTWQA  247
D. rerio      189   PICDFTPGSGFFPYCSFTNGETSPTVSMAELEHLAQNISKSHMETCQYLREELQQMTWQA  247
                    ************************************************************

C. auratus          FLQEEVESYHSKPREVMWQLCAIKITEAIQYVVEFAKRIDGFMELCQNDQIVLLKAGSLE
C. carpio     156   FLQEEVESYQSKPREVMWQLCAIKITEAIQYVVEFAKRIDGFMELCQNDQIVLLKAGSLE  215
S. grahami    249   FLQEEVECYQSKPREVMWQLCAIKITEAIQYVVEFAKRIDGFMELCQNDQIVLLKAGSLE  307
D. rerio      249   FLQEEVENYQSKPREVMWQLCAIKITEAIQYVVEFAKHIDGFMELCQNDQIVLLKAGSLE  307
                    ******* *:***************************:**********************

C. auratus          VVFIRMCRAFDPQNNTVYFDGKYAGPDVFKSLGCDDLISSVFEFAKNLCSMHLSEDEIAL
C. carpio     216   VVFIRMCRAFDPQNNTVYFDGKYAGPDVFKSLGCDDLISSVFEFAKNLCSMHLSEDEIAL  275
S. grahami    309   VVFVRMCRAFDPQNNTVYFDGKYAGHDVFKSLGCDDLISSVFEFGKSLCSMHLSEDEIAL  367
D. rerio      309   VVFVRMCRAFDPQNNTVYFDGKYAGPDVFKSLGCDDLISSVFEFGKNLCSMHLSEDEIAL  367
                    ***:******************** *****************.*.*************

C. auratus          FSAFVLMSADRSWLQEKVKVEKLQQKIQLALQHVLQKNHREDGILTKLICKVSTLRALCS
C. carpio     276   FSAFVLMSADRSWLQEKVKVEKLQQKIQLALQHVLQKNHREDGILTKLICKVSTLRALCS  335
S. grahami    369   FSAFVLMSADRSWLQEKVKVEKLQQKIQLALQHVLQKNHREDGILTKLICKVSTLRALCS  427
D. rerio      369   FSAFVLMSADRSWLQEKVKVEKLQQKIQLALQHVLQKNHREDGILTKLICKVSTLRALCS  427
                    ************************************************************

C. auratus          RHTEKLTAFKAIYPDIVRAHFPPLYKELFGSDFEQSMPVD
C. carpio     336   RHTEKLTAFKAIYPDIVRAHFPPLYKELFGSDFEQSMPVD  375
S. grahami    429   RHTEKLTAFKAIYPDIVRAHFPPLYKELFGSDFEQSMPVD  467
D. rerio      429   RHTEKLTAFKAIYPDIVRAHFPPLYKELFGSDFEQSMPVD  467
                    ***************************************
```

Figure S 1. Alignment of the deduced amino acid sequences of rev-erbβ b and rorα a of goldfish with the coincident fragments of Danio rerio (NM_131065.1 and NM_001110167.1), Cyprinus carpio (XM_019124111.1 and XM_019100303.1), and Sinocyclocheilus grahami (XM_016256081.1 and XM_016290300.1) sequences.

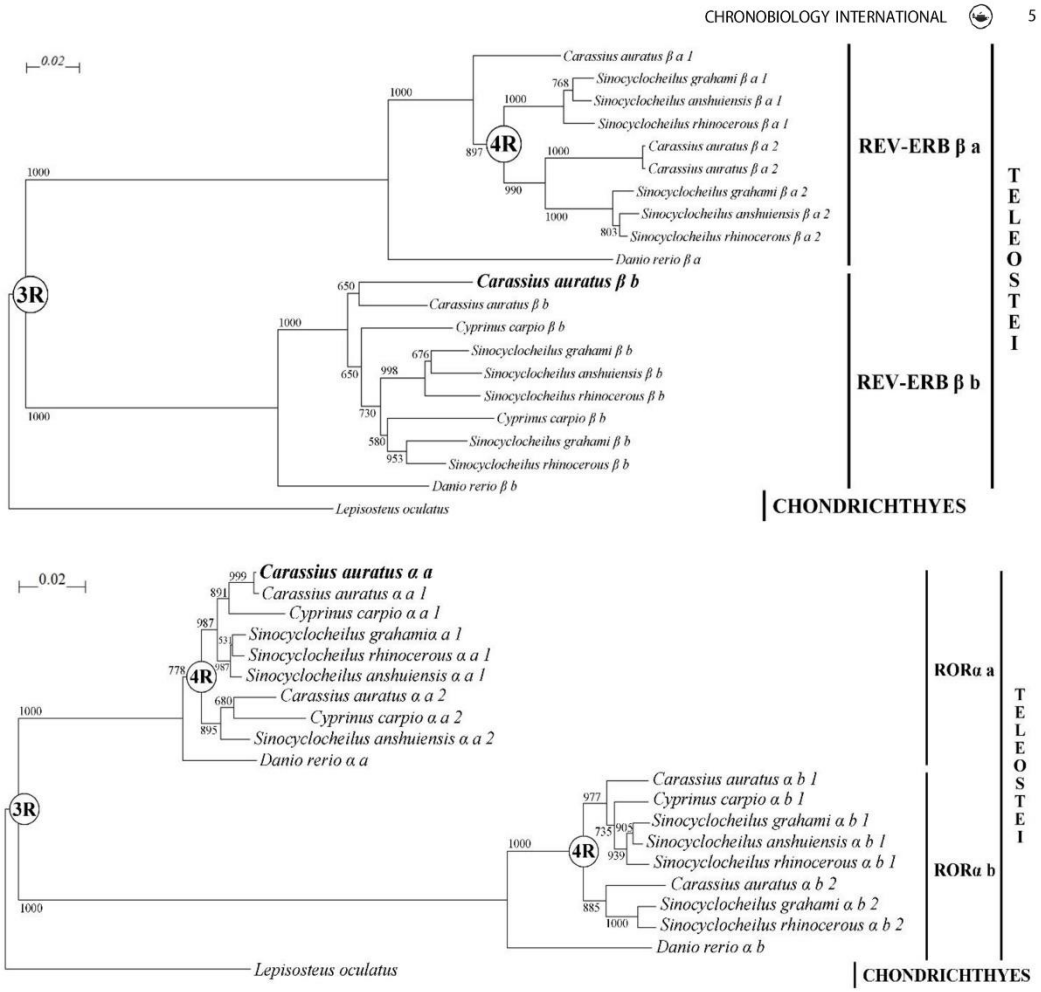


Figure S2. A phylogenetic tree showing the evolutionary relationships of the deduced amino acid sequences of goldfish REV-ERBβ b (**top**) and RORα a (**bottom**) with those of other species was inferred by the neighbor-joining method using MEGA6 (Tamura et al., 2013). The numbers at tree nodes refer to percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The scale bar indicates the average number of substitutions per position (a relative measure of evolutionary distance). The scientific name of the species used for the alignment is given on the right side of the tree, among which our sequence of goldfish is in bold. Species names and GenBank accession numbers of the sequences used for **REV-ERBβ** are as follows: *Carassius auratus*, a1, (XM_026283205.1), a2, (XM_026227800.1 and XM_026242557.1), b, (XM_026241077.1); *Cyprinus carpio*, b, (XM_019123835.1 and XM_019124111.1); *Danio rerio*, a, (NM_001130592.2), b, (NM_131065.1); *Lepisosteus oculatus*, (XM_006635767.2); *Sinocyclocheilus anshuiensis*, a1, (XM_016485802.1), a2, (XM_016464355.1), b, (XM_016502392.1); *Sinocyclocheilus grahami*, a1, (XM_016254867.1), a2, (XM_016281483.1), b, (XM_016250450.1 and XM_016256081.1); and *Sinocyclocheilus rhinocerous*, a1, (XM_016511718.1), a2, (XM_016539323.1), b, (XM_016557383.1 and XM_016527383.1). Species names and GenBank accession numbers of the sequences used for **RORα** are as follows: *Carassius auratus*, a1, (XM_026262689.1), a2, (XM_026239202.1), b1, (XM_026211918.1), b2, (XM_026216734.1); *Cyprinus carpio*, a1, (XM_019100303.1), a2, (XM_019104808.1), b1, (XM_019102978.1); *Danio rerio*, a, (NM_001110167.1), b, (NM_201067.1); *Lepisosteus oculatus*, (XM_015343697.1); *Sinocyclocheilus anshuiensis*, a1, (XM_016448481.1), a2, (XM_016505768.1), b1, (XM_016468431.1); *Sinocyclocheilus grahami*, a1, (XM_016290300.1), b1, (XM_016273167.1), b2, (XM_016282672.1); and *Sinocyclocheilus rhinocerous*, a1, (XM_016532983.1), b1, (XM_016565926.1), b2, (XM_016554123.1).

frontiers in Physiology

ORIGINAL RESEARCH
published: 05 December 2018
doi: 10.3389/fphys.2018.01749

Time-Lag in Feeding Schedule Acts as a Stressor That Alters Circadian Oscillators in Goldfish

*Miguel Gómez-Boronat, Nuria Sáiz, María J. Delgado, Nuria de Pedro and Esther Isorna**

Departamento de Genética, Fisiología y Microbiología, Unidad Docente de Fisiología Animal, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Madrid, Spain

The circadian system controls temporal homeostasis in all vertebrates. The light-dark (LD) cycle is the most important *zeitgeber* ("time giver") of circadian system, but feeding time also acts as a potent synchronizer in the functional organization of the teleost circadian system. In mammals is well known that food intake during the rest phase promotes circadian desynchrony which has been associated with metabolic diseases. However, the impact of a misalignment of LD and feeding cycles in the entrainment of fish circadian oscillators is largely unknown. The objective of this work was to investigate how a time-lag feeding alters temporal homeostasis and if this could be considered a stressor. To this aim, goldfish maintained under a 12 h light-12 h darkness were fed at mid-photophase (SF6) or mid-scotophase (SF18). Daily rhythms of locomotor activity, clock genes expression in hypothalamus, liver, and head kidney, and circulating cortisol were studied. Results showed that SF6 fish showed daily rhythms of *bmal1a* and *clock1a* in all studied tissues, being in antiphase with rhythms of *per1* genes, as expected for proper functioning clocks. The 12 h shift in scheduled feeding induced a short phase advance (4–5-h) of the clock genes daily rhythms in the hypothalamus, while in the liver the shift for clock genes expression rhythms was the same that the feeding time shift (~12 h). In head kidney, acrophases of *per* genes underwent a 12-h shift in SF18 animals, but only 6 h shift for *clock1a*. Plasma cortisol levels showed a significant daily rhythm in animals fed at SF6, but not in SF18 fish fed, which displayed higher cortisol values throughout the 24-h. Altogether, results indicate that hypothalamus, liver, and head kidney oscillate in phase in SF6 fish, but these clocks are desynchronized in SF18 fish, which could explain cortisol alterations. These data reinforce the hypothesis that the misalignment of external cues (daily photocycle and feeding time) alters fish temporal homeostasis and it might be considered a stressor for the animals.

Keywords: goldfish, hypothalamus, interrenal tissue, liver, circadian system, food intake, clock genes

OPEN ACCESS

Edited by:
Lluis Tort, Autonomous University of Barcelona, Spain

Reviewed by:
Jose Fernando Lopez-Olmeda, University of Murcia, Spain
Zhigang Shen, Huazhong Agricultural University, China

***Correspondence:**
Esther Isorna
eisornaa@bio.ucm.es

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Physiology

Received: *26 July 2018*
Accepted: *20 November 2018*
Published: *05 December 2018*

Citation:
Gómez-Boronat M, Sáiz N, Delgado MJ, de Pedro N and Isorna E (2018) Time-Lag in Feeding Schedule Acts as a Stressor That Alters Circadian Oscillators in Goldfish. Front. Physiol. 9:1749. doi: 10.3389/fphys.2018.01749

INTRODUCTION

The circadian system in vertebrates is formed by a widespread network of self-sustainable endogenous clocks located in central and peripheral tissues (Albrecht, 2012; Schibler et al., 2015; Costa et al., 2016; Isorna et al., 2017). These clocks generate circadian endogenous rhythms with a period close, but generally not equal, to 24 h, providing a temporal organization for

physiological and behavioral activities making it possible to predict environmental changes (i.e., *zeitgebers*; Albrecht, 2012; Tsang et al., 2014; Challet, 2015). The most important environmental factor that entrains circadian oscillators is the light-dark (LD) cycle, and clocks synchronized by this *zeitgeber* ("time giver" in German) are named Light-Entrainable Oscillators (LEOs; Reppert and Weaver, 2002; Mendoza and Challet, 2009). However, feeding time is also an important *zeitgeber*, especially for peripheral clocks, and clocks entrained by feeding-fasting cycles are known as Feeding-Entrainable Oscillators (FEOs; Damiola et al., 2000; Mendoza and Challet, 2009).

The circadian clocks machinery is well conserved in vertebrates and it is based on transcriptional-translational feedback loops. The positive limb of the main loop is represented by two transcription factors, CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle ARNT-Like 1), whose heterodimer binds to an E-box rich region in the promoter of the negative limb genes *period* (*per*) and *cryptochrome* (*cry*) (Gekakis et al., 1998; Nakamura et al., 2008). This binding promotes the expression of these last two clock genes, whose products PER and CRY heterodimerize in the cytoplasm and translocate into the nucleus to repress CLOCK-BMAL1 transactivation (Hastings et al., 2007; Nader et al., 2010; Schibler et al., 2015). Moreover, the CLOCK-BMAL1 heterodimer also induces the expression of genes known as clock-controlled genes (CCG), which are considered the outputs of the clock by binding to the E-boxes in their promoters (Hastings et al., 2007; Vatine et al., 2011; Albrecht, 2012). The functioning of this molecular mechanism is conserved, although several copies of these clock genes have been reported in fishes (Vatine et al., 2011; Sánchez-Bretaño et al., 2015a).

In mammals, the master pacemaker is a LEO located in the suprachiasmatic nucleus of the hypothalamus (Reppert and Weaver, 2002; Welsh et al., 2010) that controls in an hierarchical manner the rest of pacemakers widely distributed over the organisms (Dibner et al., 2010). It is evident that the organization of the circadian system in fish is less hierarchical than in mammals, since a master clock has not been clearly identified yet (Moore and Whitmore, 2014; Sánchez-Bretaño et al., 2015a; Isorna et al., 2017). Despite the greater or lesser hierarchical role of central pacemakers, evidences of the physiological relevance of peripheral circadian clocks in vertebrates are emerging. It is suggested that the entrainment of peripheral clocks by feeding-fasting cycles allows peripheral tissues to anticipate food supply, and potentially optimizing processes required for food digestion, metabolism, and energy storage and utilization (Vera et al., 2007; Lamia et al., 2008). Indeed, food intake during the rest phase promotes circadian desynchrony, which has been associated with metabolic diseases in mammals (Ferrell and Chiang, 2015; Ramirez-Plascencia et al., 2017), thus a time-lag feeding schedule can be considered a stressor that alters temporal homeostasis. In fish, feeding time is a potent *zeitgeber* for peripheral oscillators of the gastrointestinal tract (Isorna et al., 2017). In fact, feeding time affects daily locomotor activity rhythms (Aranda et al., 2001; Cavallari et al., 2011; Feliciano et al., 2011); clock genes expression in liver, gut, and encephalic tissues (López-Olmeda et al., 2009, 2010; Feliciano et al., 2011; Nisembaum et al., 2012; Tinoco et al., 2014); and daily profile of circulating cortisol (Montoya et al., 2010; Cowan et al., 2017). But a variety of results are obtained depending on species and protocols employed (Cowan et al., 2017). Nevertheless, the effect of feeding time on the clock of the interrenal tissue has not been investigated in any fish species to date, and it is unknown if this oscillator behaves as a LEO or a FEO. In fact, the paradigm of a time-lag in feeding schedule and its consequences in locomotor activity, peripheral oscillators and cortisol production has not been studied all at once and in the same species.

Therefore, the aim of this work was to study, if a time-lag in scheduled feeding alters temporal homeostasis in fish and to test its possible role as a stressor. To this end, we have studied the effects of 12 h shifted feeding schedule on daily expression of clock genes in the hypothalamus and two peripheral oscillators, the liver and the head kidney in goldfish (*Carassius auratus*). We have also investigated if this paradigm affects circulating cortisol daily rhythms as stress indicator and hepatic leptin expression as a putative output of the liver clock. The interest to study such oscillators is based on several reasons. The hypothalamus plays a key role in the control of both, energy homeostasis and the hypothalamus-pituitary-interrenal (HPI) axis, acting as an integrative core of environmental and endogenous signals. The role of the liver as a nexus between metabolism and circadian system in mammals and fish has been outlined (Albrecht, 2012; Schmutz et al., 2012; Tsang et al., 2014; Schibler et al., 2015), emphasizing this tissue as a key food-sensitive clock. Finally, the interrenal tissue (contained in the head kidney) is the main source of cortisol, which initiates the stress response (Schreck and Tort, 2016), and its daily rhythm is considered as the most robust hormonal rhythmic output in vertebrates (Isorna et al., 2017; Spencer et al., 2018).

MATERIALS AND METHODS

Animals and Housing

Goldfish (*C. auratus*) with a body weight (bw) of 24 ± 5 g were obtained from a local commercial supplier (ICA, Madrid, Spain). Fish were housed in 60 l aquaria with filtered and aerated fresh water (21 ± 2°C) under a 12 h light and 12 h darkness (12L:12D) photoperiod (lights on at 8 am, considered as *Zeitgeber* Time 0, ZT 0). Fish were fed with automatic feeders that daily delivered food pellets (1% bw; Sera Pond Biogranulat, Heinsberg, Germany) at ZT 2. Animals were acclimated during 2 weeks under these conditions before the beginning of the experiments. The experiments comply with the Guidelines of the European Union Council (UE63/2010), and the Spanish Government (RD53/2013) for the use of animals in research and were approved by the Animal Experimentation Committee of Complutense University (O.H.-UCM-25-2014), and the Community of Madrid (PROEX 107/14).

Experimental Design

Two groups of fish maintained under the same 12L:12D photoperiod (lights on at 8 a.m.) were fed with different schedules with automatic feeders to avoid the negative effects of the human feeding activities. One group (n = 36, placed in six aquaria, six fish/tank) was daily fed at mid-photophase (ZT 6, named Scheduled Feeding 6, SF6), and the other one (n = 36, placed in six aquaria) was daily fed at mid-scotophase (ZT 18, named SF18). Three weeks later, goldfish were sampled each 4 h throughout a 24 h cycle (one tank (n = 6) per sampling time at ZT 5, ZT 9, ZT 13, ZT 17, ZT 21, and ZT 1). Blood was collected from the caudal vein of anesthetized animals (tricaine methanesulfonate, MS-222, 0.14 g/l; Sigma-Aldrich, Madrid, Spain), and plasma was obtained after blood centrifugation and stored at −80°C until assay. Fish were then sacrificed by anesthetic overdose (MS-222, 0.28 g/l), and hypothalamus, head kidney, and liver were quickly collected, frozen in liquid nitrogen and stored at −80°C until analysis.

Locomotor Activity Recordings

Daily locomotor activity was recorded during the experimental period by six infrared photocells (Omron Corporation, E3S-AD12, Japan) fixed on the walls of each aquarium wall. Two photocells were located below the automatic feeder (for recording feeding-related activity), while the remaining four photocells were placed at a height of 3–9 cm above the bottom in each aquaria wall (for recording general locomotor activity). With this arrangement of photocells, we obtained reproducible actograms, more photocells increase the total amount of activity but does not affect daily profiles. Each photocell continuously emitted an infrared light beam which was interrupted each time fish swam in that zone, generating an output signal. The number of light beam interruptions was automatically registered every 10 min by a computer with specific software (Micronec, Spain). The aquaria walls were covered with opaque paper to minimize external interferences during the experiment. Data were analyzed using the chronobiology software EL TEMPS®(Prof. Antoni Díez Noguera, University of Barcelona), and actograms and periodograms were performed.

Gene Expression Analysis

Total RNA from hypothalamus, head kidney, and liver were isolated using TRI®Reagent (Sigma-Aldrich) and treated with RQ1 RNase-Free DNase (Promega, Madison, United States) according to the manufacturer's instructions. Then, 0.3 µg of total RNA was reverse transcribed into cDNA in a 25 µl reaction volume using random primers (Invitrogen, Carlsbad, United States), RNase inhibitor (Promega), and SuperScript II Reverse Transcriptase (Invitrogen). The reverse transcription reaction conditions consisted of an initial step at 25°C for 10 min, an extension at 42°C for 50 min, and a denaturalization step at 70°C for 15 min. Real-Time quantitative PCRs (RT-qPCRs) were carried out by duplicate in a CFX96 Real^{TM}-Time System (Bio-Rad Laboratories, Hercules, United States), using iTaq^{TM} Universal SYBR®Green Supermix (Bio-Rad Laboratories) using a 96-well plate loaded with 1 µl of cDNA and a final concentration of 0.5 µM of each forward and reverse primers in a final volume of 10 µl. Each PCR run included also a four-points serial standard curve, non-retrotranscribed-RNA (as positive control) and water (as negative control). The RT-qPCR cycling conditions consisted of an initial denaturation at 95°C for 30 s and 40 cycles of a two-step amplification program (95°C for 5 s and 60°C for 30 s). A melting curve was systematically monitored (temperature gradient at 0.5 C/5 s from 70 to 90°C) at the end of each run to confirm the specificity of the amplification reaction. The Gene Data Bank reference numbers and the primers (Sigma-Aldrich) sequences employed for target genes (clock genes: *per1a*,

TABLE 1 | Accession numbers of the genes and primers sequences employed in quantitative RT-qPCR studies.

Gene	Accession number		Primer sequence 5′→3′	Product (bp)
per1a	EF690698	Forward	CAGTGGCTCGA ATGAGCACCA	155
		Reverse	TGAAGACCTG CTGTCCGTTGG	
per1b	KP663726	Forward	CTCGCAGCTC CACAAACCTA	235
		Reverse	TGATCGTGCA GAAGGAGCCG	
per2a	EF690697	Forward	TTTGTCAATC CCTGGAGCCGC	116
		Reverse	AAGGATTTGC CCTCAGCCACG	
per3	EF690699	Forward	GGCTATGGCAGT CTGGCTAGTAA	130
		Reverse	CAGCACAAAAC CGCTGCAATGTC	
bmal1a	KF840401	Forward	AGATTCTGTT CGTCTCGGAG	161
		Reverse	ATCGATGAGTC GTTCCCGTG	
clock1a	KJ574204	Forward	CGATGGCAGC ATCTCTTGTGT	187
		Reverse	TCCTGGATCTG CCGCAGTTCAT	
leptin al	FJ534535	Forward	AGCTCCTCA TAGGGGATC	192
		Reverse	TAGATGTCGTT CTTTCCTTA	
ef-1α	AB056104	Forward	CCCTGGCCA CAGAGATTTCA	101
		Reverse	CAGCCTCGAA CTCACCAACA	

per period; bmal1a, brain and muscle ARNT-like 1a; clock1a circadian locomotor output cycles kaput 1a; ef-1α, elongation factor-1α.

per1b, *per2a*, *per3*, *bmal1a,* and *clock1a*; and *leptin aI)* and the reference gene (*ef-1α*) are shown in **Table 1**. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression (fold change). Data obtained were normalized to the group with the lowest expression in each gene.

Plasma Cortisol Assay

Plasma cortisol levels were determined by enzyme–linked immunosorbent assay (ELISA) using a commercial kit (Demeditec, Schleswig-Holstein, Germany), previously validated for goldfish plasma (Azpeleta et al., 2010). The lowest analytical detectable level of cortisol that can be distinguished from the zero calibrator was 3.79 ng/ml. Free cortisol values were expected to be within the range described by the manufacturer (10–800 ng/ml), therefore no dilution was necessary.

Data Analysis

The existence of significant periods in daily locomotor activity was analyzed by constructing chi-square periodograms with a significance level set at 0.05 (EL TEMPS®). A one-way ANOVA followed by the *post hoc* Student-Newman-Keuls (SNK) test was performed to compare data obtained for gene expression and cortisol levels at different sampling points (using SigmaPlot 12.0 statistics package). When necessary, data were transformed to logarithmic or square root scale to normalize and to obtain homoscedasticity. Statistical differences among groups were noted with different letters. In addition, we have performed a Mann-Whitney *U* Test for analyzing the differences between the mean of cortisol levels in fish fed at ZT 6 and ZT 18. A probability level of $p < 0.05$ was considered statistically significant in all tests. Daily (24 h) significant rhythms in gene expression and cortisol were determined by Cosinor analysis fitting the data to sinusoidal functions by the least squares method (Duggleby, 1981). The formula used was $f(t) = M+A\cos(t\pi/12-\Phi)$, where $f(t)$ is the gene expression level at a given time, the mesor (M) is the mean value, A is the sinusoidal amplitude of oscillation, t is time in hours, and Φ is the acrophase (time of peak expression). Non-linear regression allows the estimation of M, A, Φ, and their standard errors (SE), which are calculated on the residual sum of squares in the least-squares fit (Duggleby, 1981; Delgado et al., 1993). Significance of Cosinor analysis was defined by the noise/signal of amplitude calculated from the ratio *SE(A)/A* (Nisembaum et al., 2012).

RESULTS

Effects of Feeding Time on Synchronization of Locomotor Activity Daily Rhythms

Daily locomotor activity was registered during 14 days before sampling. Representative double-plotted actograms with the general locomotor activity of fed fish at ZT 6 and ZT 18 are shown in **Figures 1A,B**, respectively, while the feeding-related activity is shown in **Figure 1C** (SF6) and **Figure 1D** (SF18). General activity of SF6 goldfish displayed a diurnal significant rhythm (evidenced by a significant 24 h period; **Figure 1A**), with higher general activity during the photophase (80% of total activity). As

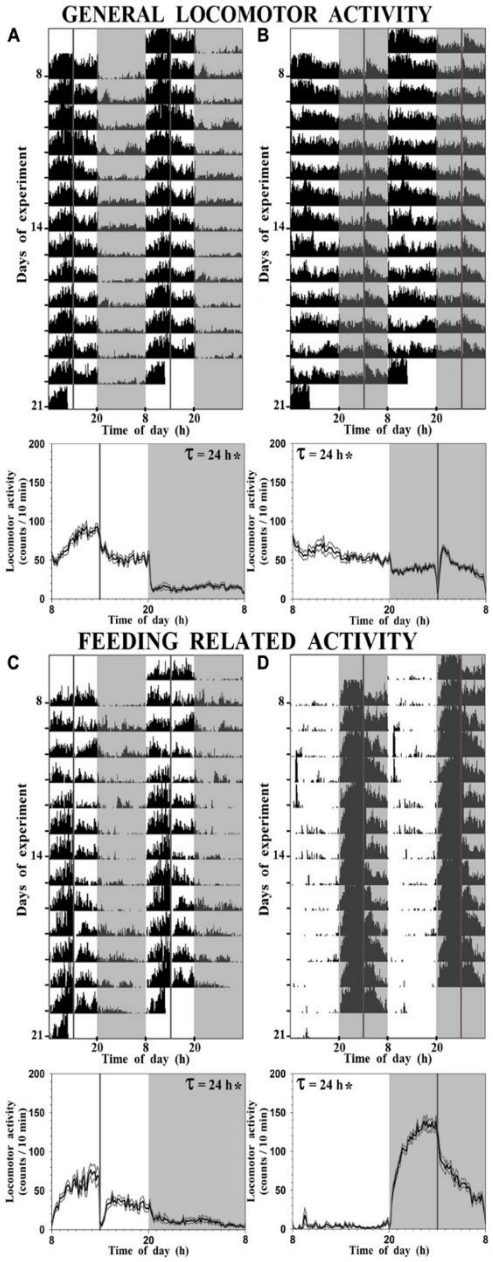


FIGURE 1 | Representative actograms and daily average waveform of goldfish maintained under a 12L:12D photoperiod and fed at ZT 6 [SF6, **(A,C)**] or at ZT 18 [SF18, **(B,D)**]. For convenient visualization, data in the actograms have been double-plotted in a time scale of 48 h. **(A,B)**, general locomotor activity; **(C,D)**, feeding-related locomotor activity. In the average waveform graphs, black line represents the mean, gray lines represent the SEM, and τ indicates the period of the rhythm when significant (*).

expected, the feeding-related activity was concentrated around 3–4 h before scheduled feeding, corresponding to the food anticipatory activity (FAA), with a significant daily rhythm with a period of 24 h (**Figure 1C**). When scheduled feeding time was shifted to the mid-scotophase, the general locomotor activity remained rhythmic (period of 24 h), but its 24 h profile was flattened (**Figure 1B**), and surprisingly general locomotor activity continued being higher during the photophase (60% of total activity). Nevertheless, fish fed at ZT 18 showed a robust FAA during the night with a significant daily rhythm (period of 24 h; **Figure 1D**).

Daily Rhythms of Clock Genes Expression in Goldfish

In the hypothalamus of SF6 animals, all studied genes exhibited significant 24 h rhythms (**Figure 2**), with acrophases of *per1* genes at the end of the dark phase (ZT 22.7 for *per1a*; **Figure 2A**) and at the light onset (ZT 1.2 for *per1b*; **Figure 2B**). These rhythmic profiles are in antiphase with those shown by *bmal1a* (ZT 11.3; **Figure 2E**) and *clock1a* (ZT 14.3; **Figure 2F**). Hypothalamic *per3* expression in the SF6 fish peaked around ZT 4 (**Figure 2D**), while the maximum expression of *per2a* occurred at midday (ZT 7.6; **Figure 2C**). The expression profiles of the clock genes in the scheduled-fed goldfish at ZT 18 also showed 24 h rhythms in the hypothalamus (**Figures 2A,B,D,E**), except for *per2a* and *clock1a*, whose rhythms were lost (**Figures 2C–F**). The shift in the scheduled feeding time from ZT6 to ZT18 advanced 4–5 h the acrophases in the case of *per1a, per1b,* and *bmal1a* genes, and 9 h for *per3* (**Figures 5A,B**) in hypothalamus.

In the head kidney, all examined clock genes showed significant daily variation in their expression in both groups of scheduled-fed goldfish (SF6 and SF18; **Figure 3**), with the exception of *per2a* and *bmal1a*, which lost their significant daily rhythmicity when scheduled feeding was shifted from midday to midnight (**Figures 3C–E**). The daily expression profiles in the head kidney of SF6 fish were broadly similar to the rhythms observed in the hypothalamus, with similar acrophases, as it can be observed in polar graphs (**Figures 5A–D**). However, a slight shift seems to exist for *per1b* and *per1a* in the head kidney of SF6 fishes compared to the hypothalamus of the same animals (**Figures 5A–C**). The amount of *per1* transcripts peaked at the early morning, which is in antiphase with the expression of *bmal1a* and *clock1a*, whose acrophases were located at the end of the light phase and beginning of the dark phase, as occurs in the hypothalamus. Thus, hypothalamic and head kidney oscillators seem to be in phase in SF6 fish. In contrast to the minor effect observed in the hypothalamus, the 12 h-shift in feeding schedule produced a complete shift (11–13 h) in *per1* and *per3* rhythms in the head kidney of goldfish, but only a 6 h advance for *clock1a*, suggesting that these negative and positive elements of the head kidney clock were not in antiphase. The expression of *per2a* showed a significant rhythm in the head kidney of SF6 but not in SF18 fish, as occurs in the hypothalamus, with similar acrophases in both tissues.

Clock genes expression in the goldfish liver displayed significant 24 h rhythms in both SF6 and SF18 fish (**Figure 4**), except for *per2a,* which did not show daily rhythmicity in any studied groups (**Figure 4C**). In SF6 animals, rhythmic profiles of clock genes expression were similar to those observed in the hypothalamus and the head kidney. The acrophases of *per1* rhythms are located at the light onset (ZT 0.7 and ZT 0.9 for *per1a* and *per1b,* respectively; **Figures 4A,B**) or the early morning (ZT 3.4 h for *per3*; **Figure 4D**), which is in antiphase with *bmal1a* (ZT 10.0) and *clock1a* genes (ZT 9.0; **Figures 4E,F**, **5E,F**). When feeding schedule was shifted from midday to midnight, all clock genes also underwent a 12 h shift in their acrophases, being moved to the LD transition in the case of *per* genes and to the light onset for *bmal1a* and *clock1a* genes (**Figures 4**, **5**). Thus, the hepatic oscillator seems to be in phase (i.e., positive elements *vs.* negative elements) in both SF6 and SF18 fishes, as in the hypothalamus, but not in the head kidney.

Comparing the clocks in the three analyzed tissues, in SF6 animals these clocks ticked at time (i.e., clock genes are in phase in the different tissues). However, acrophases of clock genes rhythms in the hypothalamus of SF18 animals were in antiphase with the hepatic ones, being the head kidney oscillator in an intermediate condition. Another different aspect of the liver oscillator, compared to the hypothalamus, and the head kidney, is referred to the amplitudes of the genes, which were much higher in the liver. In this sense, the amplitudes of *per* genes were more than 10 times higher than in the hypothalamus and about 3–5 times higher than in the head kidney in both SF6 and SF18 animals.

Daily Rhythms of Circulating Cortisol and Leptin Expression in the Liver

Circulating cortisol displayed a significant daily rhythm in goldfish fed at midday with a robust amplitude (143.8 ng/ml) and the acrophase during the scotophase (at ZT 18.9; **Figure 6A**) 6 h before lights on. By contrast, in the SF18 group this 24 h rhythmicity was fully abolished. Moreover, the SF18 fed fish showed significantly higher levels of cortisol (202.19 ± 22.78 ng/ml) than that observed in SF6 fed fish (126.95 ± 23.06 ng/ml) ($p < 0.05$, Mann-Whitney *U* Test). Hepatic *leptin aI* expression showed significant daily rhythms in both SF6 and SF18 fish (**Figure 6B**). The acrophase of *leptin aI* rhythm was found at the middle of the scotophase (ZT 17.6) in fish fed at ZT 6, while it was shifted at midday (ZT 5.8) in SF18 fish. Thus, the 12-h-shift in feeding schedule from midday to midnight induced a 12-h shift in the rhythmic expression of *leptin aI* in goldfish liver.

DISCUSSION

Results obtained clearly show that a shift in feeding schedule alters temporal homeostasis in goldfish, as it differently affects clocks (i.e., clock genes expression rhythms) in the hypothalamus, the liver, and the head kidney. In fish fed at midday, these three oscillators tick at time with similar acrophases for each gene

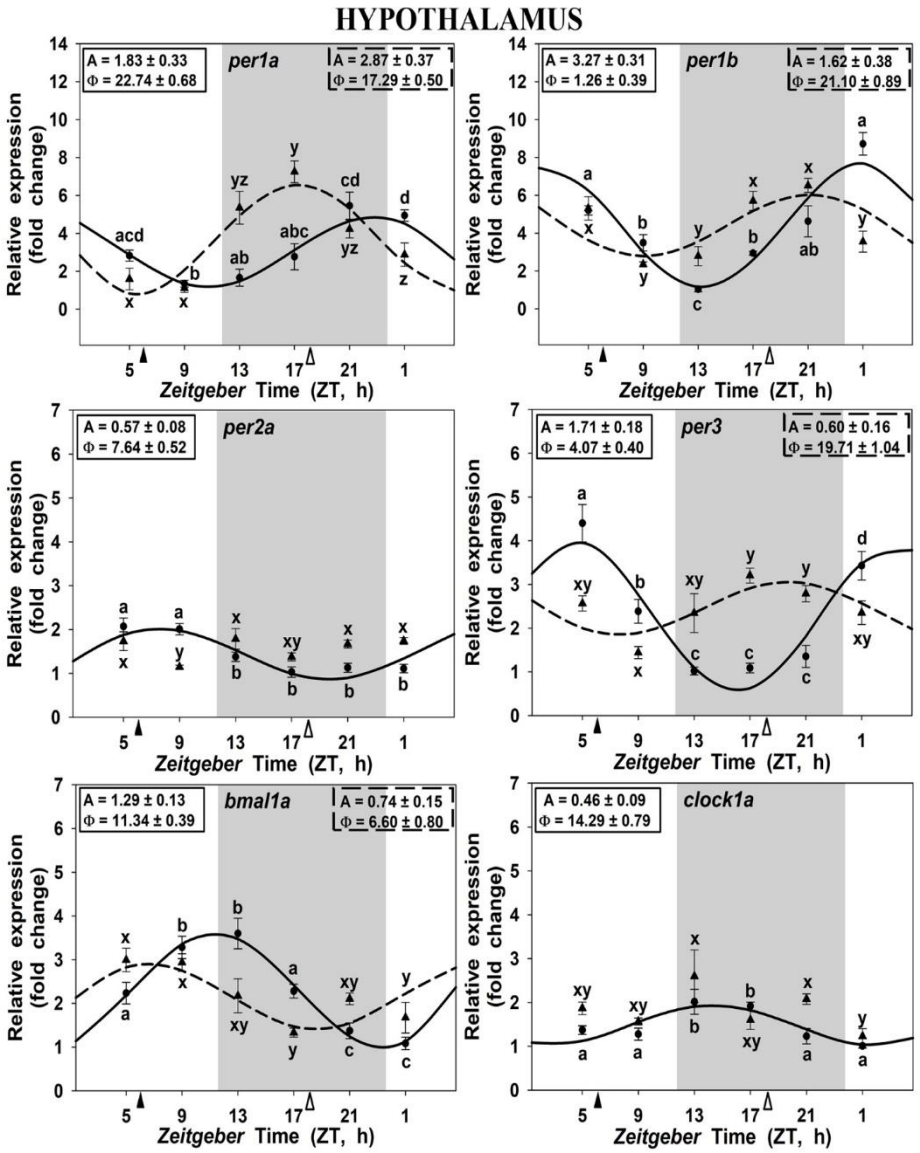


FIGURE 2 | Daily profile of clock genes expression in the hypothalamus of SF6 (●) and SF18 (▲) goldfish maintained under a 12L:12D photoperiod. Gray area indicates the dark period while feeding time is indicated by triangles in the x-axis (solid, ZT 6; white, ZT 18). Data obtained by RT-qPCR are shown as mean ± SEM ($n = 6$) in relative units ($2^{-\Delta\Delta Ct}$ method). Different letters (**a–d** in SF6 and **x–z** in SF18) indicate significant differences. When Cosinor [$SE(A)/A < 0.3$] was significant, periodic sinusoidal functions were represented as solid waves (SF6 fish) or dashed waves (SF18 fish), and amplitudes and acrophases (A and Φ, respectively) are shown at the top of the panels (SF6, left; SF18 right).

in the different tissues. However, in fish fed at mid-scotophase, daily expression rhythms of clock genes are not in phase in the different tissues, and *per1* and *clock-bmal* genes do not follow their characteristic profiles of expression in antiphase, particularly in the head kidney. Then, time-lag in feeding schedule seems to represent a stressor for the animals, since alters the temporal homeostasis, with increases in plasma cortisol and the disappearance of its daily rhythm in fish fed in the mid-scotophase.

It is widely known that food acts as a potent *zeitgeber* for circadian rhythms when restricted or provided on a periodic basis (Hara et al., 2001; Stephan, 2002). As expected, goldfish adapted their daily locomotor activity to feeding schedule; SF6 fish showed a robust FAA in the photophase while SF18 fish showed it during the scotophase. It is previously reported that a scheduled feeding under continuous light (Vera et al., 2007; Feliciano et al., 2011), at the start or the end of the photophase (Aranda et al., 2001), or at the beginning of the scotophase

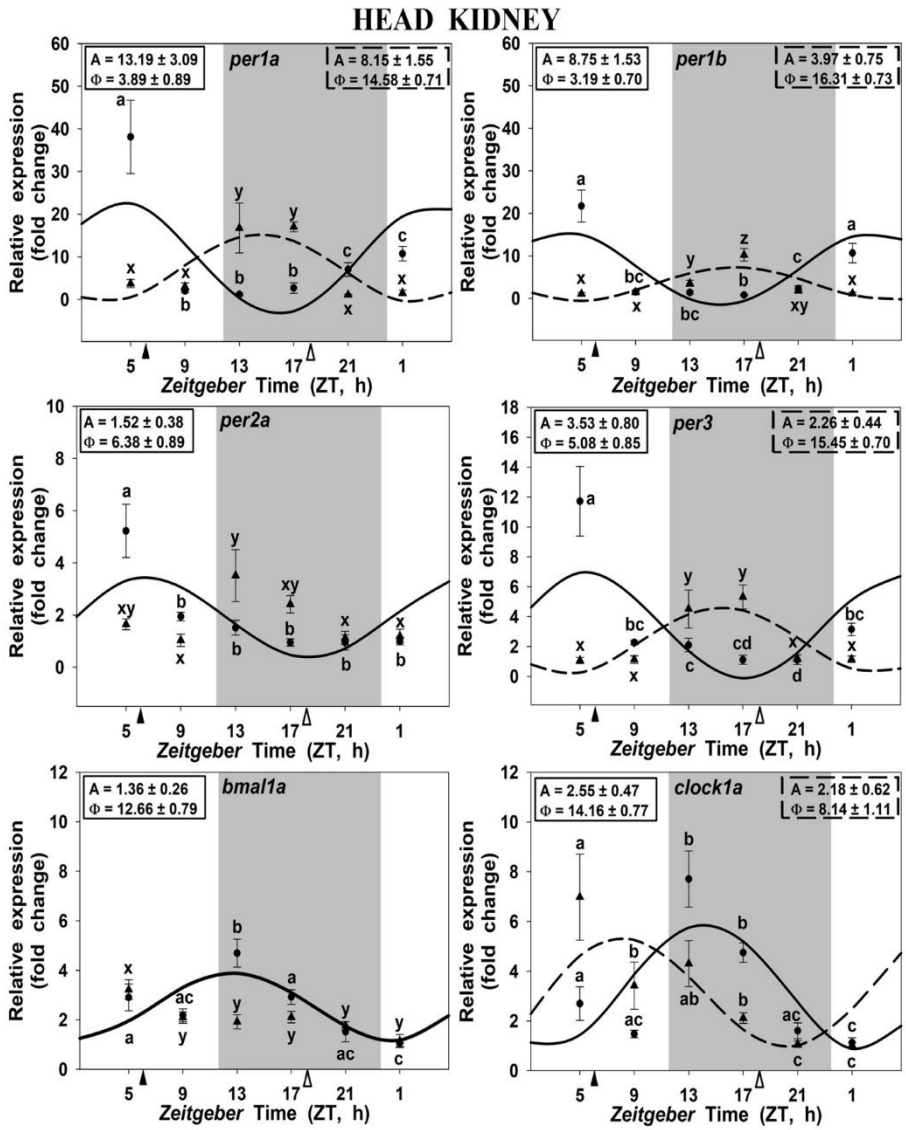


FIGURE 3 | Daily profile of clock genes expression in the head kidney of SF6 (•) and SF18 (▲) goldfish maintained under a 12L:12D photoperiod. Gray area indicates the dark period while feeding time is indicated by triangles in the x-axis (solid, ZT 6; white, ZT 18). Data obtained by RT-qPCR are shown as mean ± SEM ($n = 6$) in relative units ($2^{-\Delta\Delta Ct}$ method). Different letters (**a–c** in SF6 and **x–z** in SF18) indicate significant differences. When Cosinor [$SE(A)/A < 0.3$] was significant, periodic sinusoidal functions were represented as solid waves (SF6 fish) or dashed waves (SF18 fish), and amplitudes and acrophases (A and Φ, respectively) are shown at the top of the panels (SF6, left; SF18 right).

(Vivas et al., 2011) synchronizes daily activity to feeding time in goldfish. However, it has been also reported that if both *zeitgebers* are present, both are important (Aranda et al., 2001). In this sense, our data revealed that SF6 goldfish are clearly diurnal (80% of the activity during the photophase), but SF18 fish has not became nocturnal, since they reduce their locomotor activity during daytime but remain active through the 24 h. In fact, they continue to move more during the photophase (60%) than during the scotophase. Thus, it seems that goldfish is not as flexible as previously suggested in terms of daily activity pattern (Isorna et al., 2017). Currently, it is not possible to discern if the alteration of locomotor activity rhythm in SF18 goldfish is related to the time-lag observed in clock genes expression, or if it is due to the loss of cortisol rhythm. Further studies are needed to assess such possibilities.

In fish fed at midday (ZT 6), the *per1a* and *per1b* genes in the hypothalamus, the head kidney and the liver displayed significant daily rhythms with their acrophases at the onset of the photophase or at the end of the scotophase, in accordance with previous reports in goldfish also maintained in 12L:12D and fed

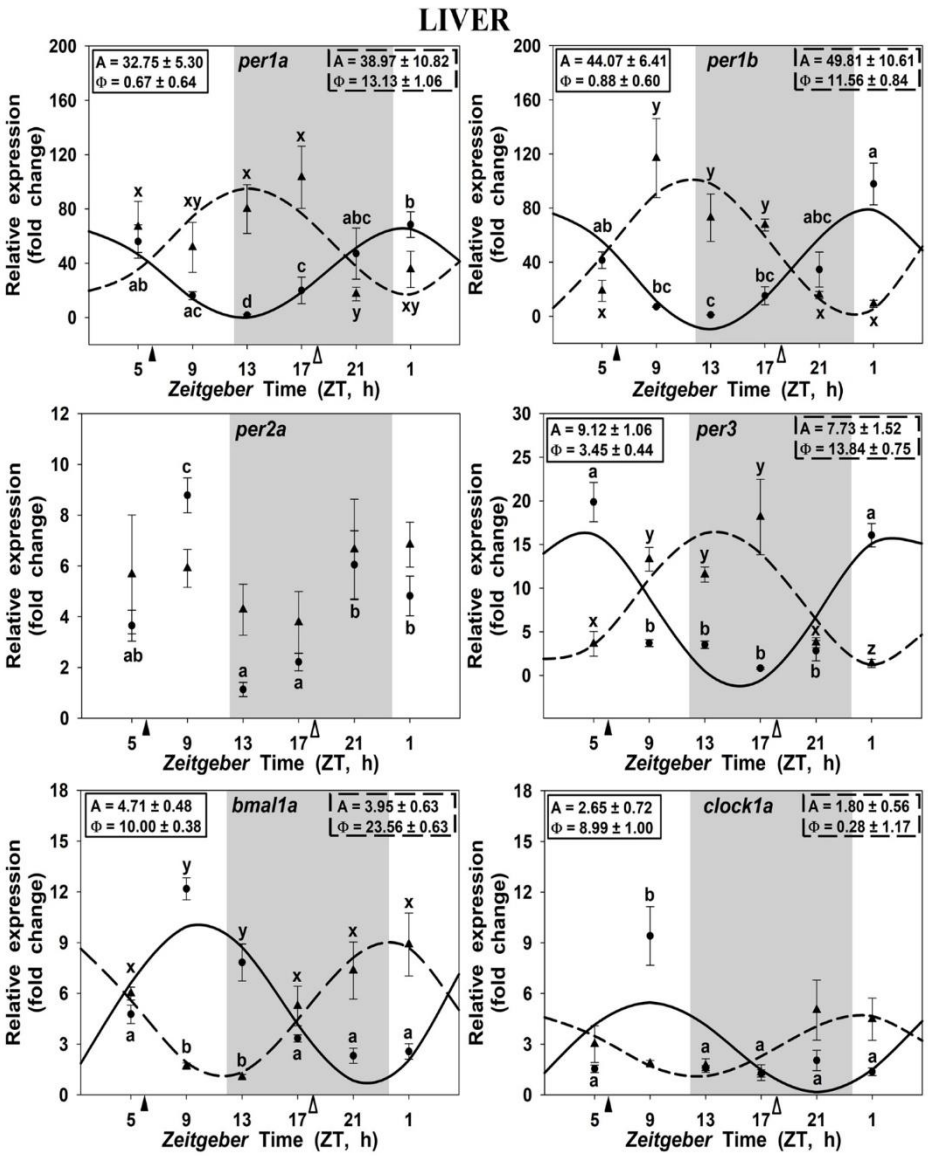


FIGURE 4 | Daily profile of clock genes expression in the liver of SF6 (●) and SF18 (▲) goldfish maintained under a 12L:12D photoperiod. Gray area indicates the dark period while feeding time is indicated by triangles in the x-axis (solid, ZT 6; white, ZT 18). Data obtained by RT-qPCR are shown as mean ± SEM ($n = 6$) in relative units ($2^{-\Delta\Delta Ct}$ method). Different letters (**a–c** in SF6 and **x–z** in SF18) indicate significant differences. When Cosinor [$SE(A)/A < 0.3$] was significant, periodic sinusoidal functions were represented as solid waves (SF6 fish) or dashed waves (SF18 fish), and amplitudes and acrophases (A and Φ, respectively) are shown at the top of the panels (SF6, left; SF18 right).

during the photophase at ZT 2 (Velarde et al., 2009; Nisembaum et al., 2012; Sánchez-Bretaño et al., 2015b). Similarly, a *per1* peak around the dark-light transition has been also reported in other teleosts, as zebrafish brain (*Danio rerio*; Sanchez and Sanchez-Vazquez, 2009; Vatine et al., 2011), European sea bass brain and liver (*Dicentrarchus labrax*; Sánchez et al., 2010), rainbow trout hypothalamus (*Oncorhynchus mykiss*; Patiño et al., 2011), Senegalese sole retina and optic tectum (*Solea senegalensis*; Martín-Robles et al., 2012), or Nile tilapia brain (*Oreochromis niloticus*; Costa et al., 2016). All these findings support the hypothesis that *per1* genes anticipate the light arrival in fish under these conditions (Isorna et al., 2017). Moreover, the clock genes of the positive limb of the loop (*bmal1a* and *clock1a*) were in antiphase with the negative limb genes (*per*) in these three tissues, showing their acrophases almost in the LD interphase, as previously reported in goldfish (Nisembaum et al., 2012), and other fish species under a LD photocycle (Patiño et al., 2011; Vatine et al., 2011; Martín-Robles et al., 2012; Costa et al., 2016).

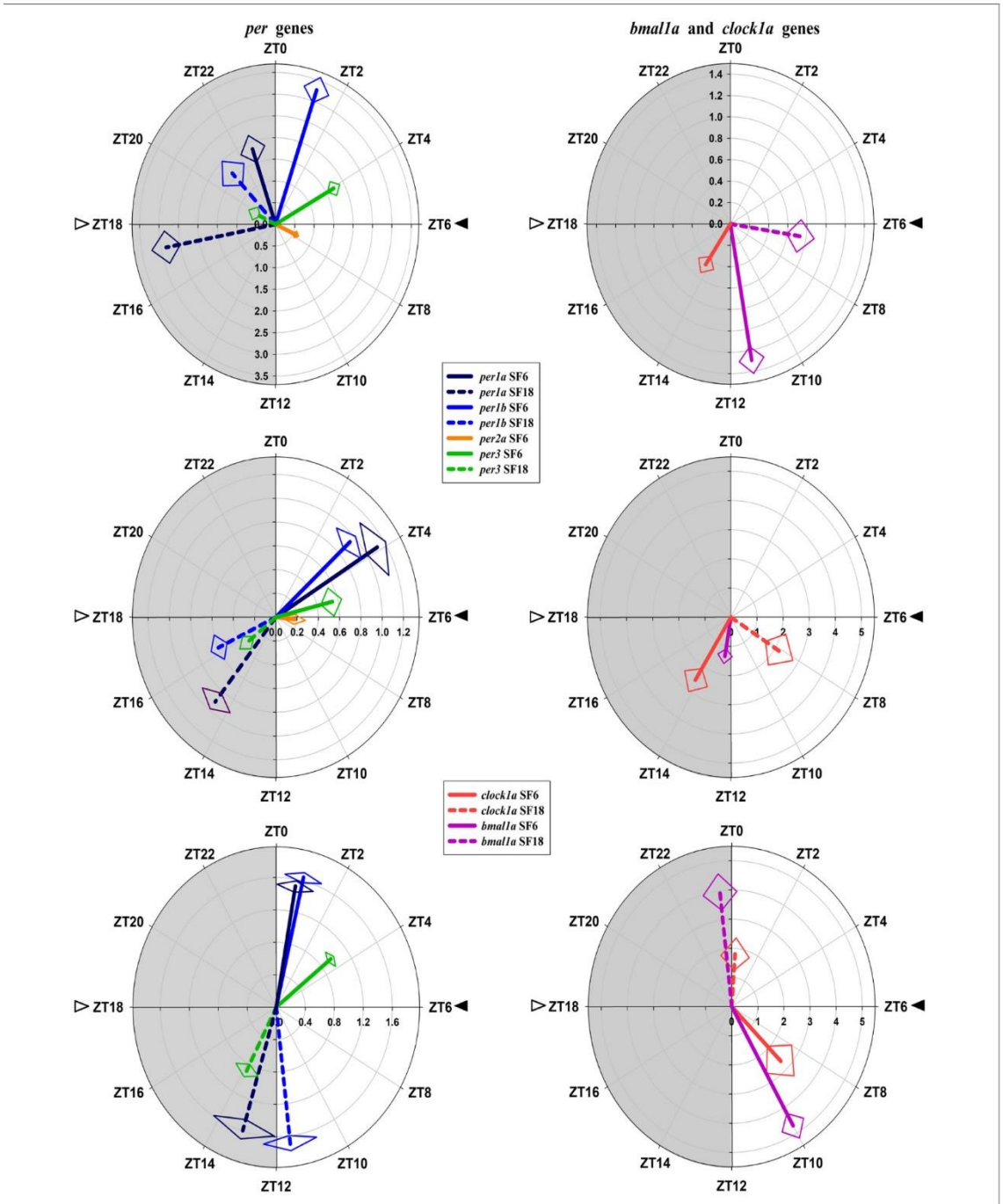


FIGURE 5 | Polar representations of parameters defining clock genes rhythms. **(A,B)** hypothalamus, **(C,D)** head kidney, **(E,F)** liver. The length of the vector (radial axis) indicates the value of the amplitude [fold change of relative expression, **C,E** in logarithmic scale]. The angular position indicates the acrophase (ZT, zeitgeber time). The SE of these two parameters is represented by the rhombus at the end of each vector.

Is feeding time able to modify such clock genes rhythmicity? As previously mentioned, food acts as a potent *zeitgeber* nor only for circadian activity rhythms (Aranda et al., 2001; Stephan, 2002; López-Olmeda et al., 2009; Refinetti, 2015) but also for clock synchronization (Damiola et al., 2000; Feliciano et al., 2011; Nisembaum et al., 2012) in mammals and fish. Our findings revealed that feeding time exerts different effects on clock genes expression at central and peripheral levels. In the hypothalamus,

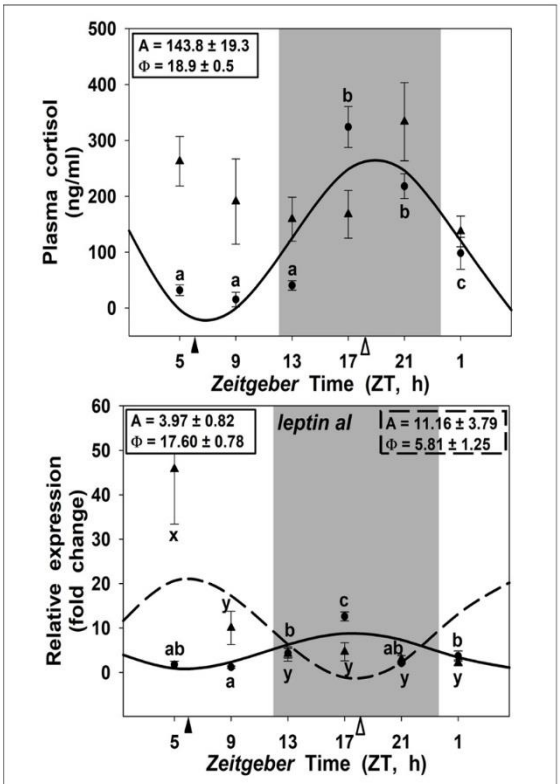


FIGURE 6 | Daily profiles of plasma cortisol **(A)** and *leptin-al* expression in the liver **(B)** of SF6 (•) and SF18 (▲) goldfish maintained under a 12L:12D photoperiod. Gray area indicates dark period and feeding time is indicated by triangles in the x-axis (solid, ZT 6; white, ZT 18). Data are shown as mean ± SEM ($n = 6$). Different letters (**a–c** in SF6, and **x–z** in SF18) indicate significant differences. When Cosinor [$SE(A)/A < 0.3$] was significant, periodic sinusoidal functions were represented as solid waves (SF6 fish) or dashed waves (SF18 fish), and amplitudes and acrophases (A and Φ, respectively) are shown at the top of the panels (SF6, left; SF18 right).

a 12 h shift in the feeding schedule (adjusting the feeding time at the mid-scotophase) induced a minor shifting of 4–5 h in the acrophases of the target genes (except *per2a* as expected and below discussed), in agreement with previous reports in the European sea bream (*Sparus aurata*; (Vera et al., 2013), and the Nile tilapia brain (Costa et al., 2016). These findings indicate that feeding time is able to induce a slight displacement of the acrophases, but the LD cycle seems to be the main synchronizer of the rhythmic expression of hypothalamic clock genes, as previously suggested (Hara et al., 2001; Sanchez and Sanchez-Vazquez, 2009; Feliciano et al., 2011; Nisembaum et al., 2012; Tinoco et al., 2014). Interestingly, the amplitudes of the central clock genes were diminished when the food was supplied at midnight (except for *per1a*), suggesting that feeding-fasting cycles enhance LD driven-daily rhythms, in agreement with previous reports (Sánchez-Bretaño et al., 2015a).

It is worthy to highlight the case of *per2a*, the only gene that did not change its expression pattern in any of the three studied tissues when feeding time was shifted. Previous reports have shown that *per2a* displayed a rhythmic expression in some central and peripheral tissues of goldfish, under a LD cycle with acrophases at midday (Velarde et al., 2009; Nisembaum et al., 2012), as in sea bass brain (Herrero and Lepesant, 2014). Such rhythms usually disappear in constant conditions, light or darkness (Feliciano et al., 2011; Nisembaum et al., 2012; Vera et al., 2013), showing that *per2a* rhythmicity is strongly dependent of the LD cycle. Indeed, it is well-known that *per2a* is a light-induced gene with a key role in the molecular mechanism that entrains the LEOs in zebrafish (Vatine et al., 2011; Moore and Whitmore, 2014; Ben-Moshe et al., 2014; Ceinos et al., 2018). Our results support this role of *per2a* as a light-dependent clock gene also in goldfish.

A substantial finding is the 12 h shifting in the acrophases of all hepatic clock genes when feeding time was shifted 12 h (from midday to midnight). Unlike in the hypothalamus, amplitudes of all rhythms shown by the different clock genes in the liver were not significantly affected by feeding time. Vera et al. (2013) obtained comparable results, reporting a 6–7 h shifting in the liver of sea bream fed at mid-photophase compared to fish fed at the mid-scotophase. All these data point out that feeding time is a synchronizer powerful than the LD cycle in the liver, as it is previously proposed in mammals (Damiola et al., 2000; Stokkan et al., 2001; Kornmann et al., 2007). This conclusion was also suggested by Feliciano et al. (2011), who demonstrate significant rhythms for clock gene expression driven by the last meal, independently of previous feeding approaches (random or scheduled feeding). Therefore, the hepatic clock might be a peripheral FEO in goldfish. In terms of adaptation to the new scheduled feeding, the shift in clock genes expression could be an advantage for the animal physiology. However, overt rhythms (i.e., outputs of the circadian system) are complex and usually dependent of more than one oscillator. Thus, although liver clock genes are synchronized to receive food at mid-scotophase, metabolic rhythms could not be adapted. In this sense, lipid metabolism rhythmicity is linked to the LD cycle, independently of feeding time in zebrafish and sea bream liver (Paredes et al., 2014, 2015), although feeding time drives clock genes oscillations in the last species (Vera et al., 2013). Surprisingly, our results show that hepatic leptin expression rhythms match with clock genes expression rhythms in liver, and the acrophase is 12 h shifted in SF6 compared to SF18 animals. This suggests that maybe not all of the metabolic outputs are driven by the same *zeitgebers* in the liver of goldfish.

Regarding the head kidney, fish fed at midday exhibit significant daily rhythms in the expression of all clock genes, with genes of the positive and negative limbs of the loop in antiphase (except *per2a*, as above discussed), confirming the existence of a functional clock in this tissue, as in the adrenal gland of mammals (Son et al., 2008; Kwon et al., 2011). Even though, the interrenal tissue of goldfish is not directly related to the gastrointestinal system, feeding time seems to play an important role on its synchronization, since the expression of *per1* genes had a peak just before the expected feeding time in both experimental groups (at ZT∼4 when food was provided at ZT 6, and at ZT∼15 when provided at ZT 18). Hence, the 12 h time-lag in the feeding time shifted the rhythmic expression pattern of *per1* genes, similarly

as the liver's response. This is not surprising, given that several peripheral clocks appear to be entrained by food in mammals (Albrecht, 2012) and in fish (López-Olmeda et al., 2010; Feliciano et al., 2011). For instance, food intake has been proven to be a potent synchronizer not only for the liver (Damiola et al., 2000; Stokkan et al., 2001; Kornmann et al., 2007), but also for the heart (Schibler et al., 2003; Mukherji et al., 2015) in mammals. In fish, meal time synchronizes the expression of clock genes in posterior intestine and liver of goldfish (Feliciano et al., 2011; Nisembaum et al., 2012; Tinoco et al., 2014), as well as in heart and fin of zebrafish (Cavallari et al., 2011). These evidences suggest that the feeding schedule has an essential role on the organization of the circadian system in vertebrates, beyond exclusively regulating digestive functions. Although it clearly seems that the interrenal tissue of midday-fed fish is a functional circadian clock, the fact that *clock1a* is not in antiphase with *per1* genes, and *bmal1a* lost its rhythmicity in goldfish fed at mid-scotophase, calls into question the functionality of the clock under this time-lag condition, and support that temporal homeostasis in SF18 animals is altered. Then, the time-lag in feeding schedule may be a stressor for goldfish.

The better adaptation of SF6 fish compared to SF18 is also supported by cortisol results. Our results demonstrate the existence of a daily cortisol rhythm in fish fed at midday, with a peak 5 h before the light onset, which correlates with the functional interrenal clock observed in this group. Conversely, animals fed at the mid-scotophase did not show a daily cortisol rhythm, owing to the fact that the basal levels of this hormone are constantly elevated, being 10 times higher than the basal levels found in midday-fed fish. Such cortisol increase in SF18 fish could be a response to a stressful situation, such as the conflict between environmental cues (light/dark cycle and meal time), that mismatches the phase of hypothalamic, hepatic, and interrenal oscillators. This alteration of circulating cortisol might be due to an altered functionality of the interrenal clock in fish fed at mid-scotophase, in agreement with the hypothesis (under debate) that a local functional clock in the interrenal tissue is necessary to maintain cortisol daily rhythms. In this sense, it is suggested that the adrenal clock could influence the circadian changes in circulating glucocorticoids in mammals (Oster et al., 2006). In fact, fish, and mammals are able to maintain daily cortisol rhythms after an hypophysectomy and in absence of cyclic ACTH levels (Srivastava and Meier, 1972; Meier, 1976), and adrenal clock genes maintain their cyclic expression in rats without a functional hypophysis (Fahrenkrug et al., 2008).

In summary, a time-lag in feeding schedule mismatches clock genes expression in the hypothalamus, the liver, and the interrenal tissue. The increment in cortisol values and the loss of its daily rhythmicity in goldfish fed at mid-scotophase could indicate that these fish are under a stressor. Thus, our results show that the loss of temporal homeostasis can negatively affect the physiology in goldfish and the underlying links between clocks and functional outputs deserve to be explored.

AUTHOR CONTRIBUTIONS

MG-B, NdP, and EI conceived and designed the experiments. MG-B, NS, and EI analyzed the samples. All authors participated in sampling animals, interpreted findings, drafted, and revised the manuscript.

FUNDING

This study was supported by Spanish Ministerio de Economía y Competitividad project (MINECO, AGL2016-74857-C3-2-R). MG-B was a predoctoral fellow from Spanish MINECO (BES-2014-068103). The authors belong to the Fish Welfare and Stress Network (MINECO, AGL2016-81808-REDT).

REFERENCES

Albrecht, U. (2012). Timing to perfection: the biology of central and peripheral circadian clocks. *Neuron* 74, 246–260. doi: 10.1016/j.neuron.2012.04.006

Aranda, A., Madrid, J. A., and Sánchez-Vázquez, F. J. (2001). Influence of light on feeding anticipatory activity in goldfish. *J. Biol. Rhythms* 16, 50–57. doi: 10.1177/074873040101600106

Azpeleta, C., Martínez-Álvarez, R. M., Delgado, M. J., Isorna, E., and de Pedro, N. (2010). Melatonin reduces locomotor activity and circulating cortisol in goldfish. *Horm. Behav.* 57, 323–329. doi: 10.1016/j.yhbeh.2010.01.001

Ben-Moshe, Z., Alon, S., Mracek, P., Faigenbloom, L., Tovin, A., Vatine, G. D., et al. (2014). The light-induced transcriptome of the zebrafish pineal gland reveals complex regulation of the circadian clockwork by light. *Nucleic Acids Res.* 42, 3750–3767. doi: 10.1093/nar/gkt1359

Cavallari, N., Frigato, E., Vallone, D., Fröhlich, N., Lopez-Olmeda, J. F., Foà, A., et al. (2011). A blind circadian clock in cavefish reveals that opsins mediate peripheral clock photoreception. *PLoS Biol.* 9:e1001142. doi: 10.1371/journal.pbio.1001142

Ceinos, R. M., Frigato, E., Pagano, C., Fröhlich, N., Negrini, P., Cavallari, N., et al. (2018). Mutations in blind cavefish target the light-regulated circadian clock gene, period 2. *Sci. Rep.* 8:8754. doi: 10.1038/s41598-018-27080-2

Challet, E. (2015). Keeping circadian time with hormones. *Diabetes Obes. Metab.* 17, 76–83. doi: 10.1111/dom.12516

Costa, L. S., Serrano, I., Sánchez-Vázquez, F. J., and López-Olmeda, J. F. (2016). Circadian rhythms of clock gene expression in Nile tilapia (*Oreochromis niloticus*) central and peripheral tissues: influence of different lighting and feeding conditions. *J. Comp. Physiol. B* 186, 775–785. doi: 10.1007/s00360-016-0989-x

Cowan, M., Azpeleta, C., and López-Olmeda, J. F. (2017). Rhythms in the endocrine system of fish: a review. *J. Comp. Physiol. B* 187, 1057–1089. doi: 10.1007/s00360-017-1094-5

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., and Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev.* 14, 2950–2961. doi: 10.1101/gad.183500

Delgado, M. J., Alonso-Gómez, A. L., Gancedo, B., de Pedro, N., Valenciano, A. I., and Alonso-Bedate, M. (1993). Serotonin N-Acetyltransferase (NAT) activity and Melatonin levels in the frog retina are not correlated during the seasonal cycle. *Gen. Comp. Endocrinol.* 92, 143–150. doi: 10.1006/gcen.1993.1151

Dibner, C., Schibler, U., and Albrecht, U. (2010). The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu. Rev. Physiol.* 72, 517–549. doi: 10.1146/annurev-physiol-021909-135821

Duggleby, R. G. (1981). A nonlinear regression program for small computers. *Anal. Biochem.* 110, 9–18. doi: 10.1016/0003-2697(81)90104-4

Fahrenkrug, J., Hannibal, J., and Georg, B. (2008). Diurnal rhythmicity of the canonical clock genes Per1, Per2 and Bmal1 in the rat adrenal gland is unaltered

after hypophysectomy. *J. Neuroendocrinol.* 20, 323–329. doi: 10.1111/j.1365-2826.2008.01651.x

Feliciano, A., Vivas, Y., de Pedro, N., Delgado, M. J., Velarde, E., and Isorna, E. (2011). Feeding time synchronizes clock gene rhythmic expression in brain and liver of goldfish (Carassius auratus). *J. Biol. Rhythms* 26, 24–33. doi: 10.1177/0748730410388600

Ferrell, J. M., and Chiang, J. Y. L. (2015). Circadian rhythms in liver metabolism and disease. *Acta Pharm. Sin. B* 5, 113–122. doi: 10.1016/j.apsb.2015.01.003

Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., et al. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. *Science* 280, 1564–1569. doi: 10.1126/science.280.5369.1564

Hara, R., Wan, K., Wakamatsu, H., Aida, R., Moriya, T., Akiyama, M., et al. (2001). Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. *Genes Cells* 6, 269–278. doi: 10.1046/j.1365-2443.2001.00419.x

Hastings, M., O'Neill, J. S., and Maywood, E. S. (2007). Circadian clocks: regulators of endocrine and metabolic rhythms. *J. Endocrinol.* 195, 187–198. doi: 10.1677/JOE-07-0378

Herrero, M. J., and Lepesant, J. M. J. (2014). Daily and seasonal expression of clock genes in the pituitary of the European sea bass (*Dicentrarchus labrax*). *Gen. Comp. Endocrinol.* 208, 30–38. doi: 10.1016/j.ygcen.2014.08.002

Isorna, E., de Pedro, N., Valenciano, A. I., Alonso-Gómez, ÁL., and Delgado, M. J. (2017). Interplay between the endocrine and circadian systems in fishes. *J. Endocrinol* 232, R141–R159. doi: 10.1530/JOE-16-0330

Kornmann, B., Schaad, O., Reinke, H., Saini, C., and Schibler, U. (2007). Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators. *Cold Spring Harb. Symp. Quant. Biol.* 72, 319–330. doi: 10.1101/sqb.2007.72.041

Kwon, I., Choe, H. K., Son, G. H., and Kim, K. (2011). Mammalian molecular clocks. *Exp. Neurobiol.* 20, 18–28. doi: 10.5607/en.2011.20.1.18

Lamia, K. A., Storch, K.-F., and Weitz, C. J. (2008). Physiological significance of a peripheral tissue circadian clock. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15172–15177. doi: 10.1073/pnas.0806717105

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

López-Olmeda, J. F., Montoya, A., Oliveira, C., and Sánchez-Vázquez, F. J. (2009). Synchronization to light and restricted-feeding schedules of behavioral and humoral daily rhythms in gilthead sea bream (*Sparus aurata*). *Chronobiol. Int.* 26, 1389–1408. doi: 10.3109/07420520903421922

López-Olmeda, J. F., Tartaglione, E. V., Iglesia, H. O., and de la Sánchez-Vázquez, F. J. (2010). Feeding entrainment of food-anticipatory activity and per1 expression in the brain and liver of zebrafish under different lighting and feeding conditions. *Chronobiol. Int.* 27, 1380–1400. doi: 10.3109/07420528.2010.501926

Martín-Robles, ÁJ., Whitmore, D., Sánchez-Vázquez, F. J., Pendón, C., and Muñoz-Cueto, J. A. (2012). Cloning, tissue expression pattern and daily rhythms of Period1, Period2, and Clock transcripts in the flatfish Senegalese sole, *Solea senegalensis*. *J. Comp. Physiol. B* 182, 673–685. doi: 10.1007/s00360-012-0653-z

Meier, A. H. (1976). Daily variation in concentration of plasma corticosteroid in hypophysectomized rats. *Endocrinology* 98, 1475–1479. doi: 10.1210/endo-98-6-1475

Mendoza, J., and Challet, E. (2009). Brain clocks: from the suprachiasmatic nuclei to a cerebral network. *Neurosci. Rev. J. Bringing Neurobiol. Neurol. Psychiatry* 15, 477–488. doi: 10.1177/1073858408327808

Montoya, A., López-Olmeda, J. F., Garayzar, A. B. S., and Sánchez-Vázquez, F. J. (2010). Synchronization of daily rhythms of locomotor activity and plasma glucose, cortisol and thyroid hormones to feeding in *Gilthead seabream* (*Sparus aurata*) under a light–dark cycle. *Physiol. Behav.* 101, 101–107. doi: 10.1016/j.physbeh.2010.04.019

Moore, H. A., and Whitmore, D. (2014). Circadian rhythmicity and light sensitivity of the zebrafish brain. *PLoS One* 9:e86176. doi: 10.1371/journal.pone.0086176

Mukherji, A., Kobiita, A., and Chambon, P. (2015). Shifting the feeding of mice to the rest phase creates metabolic alterations, which, on their own, shift the peripheral circadian clocks by 12 hours. *Proc. Natl. Acad. Sci. U.S.A.* 112, E6683–E6690. doi: 10.1073/pnas.1519735112

Nader, N., Chrousos, G. P., and Kino, T. (2010). Interactions of the circadian CLOCK system and the HPA axis. *Trends Endocrinol. Metab. TEM* 21, 277–286. doi: 10.1016/j.tem.2009.12.011

Nakamura, K., Inoue, I., Takahashi, S., Komoda, T., and Katayama, S. (2008). Cryptochrome and Period proteins are regulated by the CLOCK/BMAL1 gene: crosstalk between the PPARs/RXRα-regulated and CLOCK/BMAL1-regulated systems. *PPAR Res.* 2008:348610. doi: 10.1155/2008/348610

Nisembaum, L. G., Velarde, E., Tinoco, A. B., Azpeleta, C., de Pedro, N., Alonso-Gómez, A. L., et al. (2012). Light-dark cycle and feeding time differentially entrains the gut molecular clock of the goldfish (*Carassius auratus*). *Chronobiol. Int.* 29, 665–673. doi: 10.3109/07420528.2012.686947

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., et al. (2006). The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metab.* 4, 163–173. doi: 10.1016/j.cmet.2006.07.002

Paredes, J. F., López-Olmeda, J. F., Martínez, F. J., and Sánchez-Vázquez, F. J. (2015). Daily rhythms of lipid metabolic gene expression in zebra fish liver: response to light/dark and feeding cycles. *Chronobiol. Int.* 32, 1438–1448. doi: 10.3109/07420528.2015.1104327

Paredes, J. F., Vera, L. M., Martinez-Lopez, F. J., Navarro, I., and Vázquez, F. J. S. (2014). Circadian rhythms of gene expression of lipid metabolism in Gilthead sea bream liver: synchronisation to light and feeding time. *Chronobiol. Int.* 31, 613–626. doi: 10.3109/07420528.2014.881837

Patiño, M. A. L., Rodríguez-Illamola, A., Conde-Sieira, M., Soengas, J. L., and Míguez, J. M. (2011). Daily rhythmic expression patterns of clock1a, bmal1, and per1 genes in retina and hypothalamus of the rainbow trout, *Oncorhynchus mykiss*. *Chronobiol. Int.* 28, 381–389. doi: 10.3109/07420528.2011.566398

Ramirez-Plascencia, O. D., Saderi, N., Escobar, C., and Salgado-Delgado, R. C. (2017). Feeding during the rest phase promotes circadian conflict in nuclei that control energy homeostasis and sleep–wake cycle in rats. *Eur. J. Neurosci.* 45, 1325–1332. doi: 10.1111/ejn.13563

Refinetti, R. (2015). Comparison of light, food, and temperature as environmental synchronizers of the circadian rhythm of activity in mice. *J. Physiol. Sci.* 65, 359–366. doi: 10.1007/s12576-015-0374-7

Reppert, S. M., and Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature* 418, 935–941. doi: 10.1038/nature00965

Sánchez, J. A., Madrid, J. A., and Sánchez-Vázquez, F. J. (2010). Molecular cloning, tissue distribution, and daily rhythms of expression of per1 gene in European sea bass (*Dicentrarchus labrax*). *Chronobiol. Int.* 27, 19–33. doi: 10.3109/07420520903398633

Sanchez, J. A., and Sanchez-Vazquez, F. J. (2009). Feeding entrainment of daily rhythms of locomotor activity and clock gene expression in zebrafish brain. *Chronobiol. Int.* 26, 1120–1135. doi: 10.3109/07420520903232092

Sánchez-Bretaño, A., Alonso-Gómez, ÁL., Delgado, M. J., and Isorna, E. (2015a). The liver of goldfish as a component of the circadian system: integrating a network of signals. *Gen. Comp. Endocrinol.* 221, 213–216. doi: 10.1016/j.ygcen.2015.05.001

Sánchez-Bretaño, A., Gueguen, M.-M., Cano-Nicolau, J., Kah, O., Alonso-Gómez, ÁL., Delgado, M. J., et al. (2015b). Anatomical distribution and daily profile of gper1b gene expression in brain and peripheral structures of goldfish (*Carassius auratus*). *Chronobiol. Int.* 32, 889–902. doi: 10.3109/07420528.2015.1049615

Schibler, U., Gotic, I., Saini, C., Gos, P., Curie, T., Emmenegger, Y., et al. (2015). Clock-talk: interactions between central and peripheral circadian oscillators in mammals. *Cold Spring Harb. Symp. Quant. Biol.* 80, 223–232. doi: 10.1101/sqb.2015.80.027490

Schibler, U., Ripperger, J., and Brown, S. A. (2003). Peripheral circadian oscillators in mammals: time and food. *J. Biol. Rhythms* 18, 250–260. doi: 10.1177/0748730403018003007

Schmutz, I., Albrecht, U., and Ripperger, J. A. (2012). The role of clock genes and rhythmicity in the liver. *Mol. Cell. Endocrinol.* 349, 38–44. doi: 10.1016/j.mce.2011.05.007

Schreck, C. B., and Tort, L. (2016). "Chapter 1 - The concept of stress in fish," in *Fish Physiology Biology of Stress in Fish*, eds C. B. Schreck, L. Tort, A. P. Farrell, and C. J. Brauner (Cambridge, MA: Academic Press), 1–34. doi: 10.1016/B978-0-12-802728-8.00001-1

Son, G. H., Chung, S., Choe, H. K., Kim, H.-D., Baik, S.-M., Lee, H., et al. (2008). Adrenal peripheral clock controls the autonomous circadian rhythm of

glucocorticoid by causing rhythmic steroid production. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20970–20975. doi: 10.1073/pnas.0806962106

Spencer, R. L., Chun, L. E., Hartsock, M. J., and Woodruff, E. R. (2018). Glucocorticoid hormones are both a major circadian signal and major stress signal: how this shared signal contributes to a dynamic relationship between the circadian and stress systems. *Front. Neuroendocrinol.* 49:52–71. doi: 10.1016/j.yfrne.2017.12.005

Srivastava, A. K., and Meier, A. H. (1972). Daily variation in concentration of cortisol in plasma in intact and hypophysectomized gulf killifish. *Science* 177, 185–187. doi: 10.1126/science.177.4044.185

Stephan, F. K. (2002). The "Other" circadian system: food as a zeitgeber. *J. Biol. Rhythms* 17, 284–292. doi: 10.1177/074873040201700402

Stokkan, K.-A., Yamazaki, S., Tei, H., Sakaki, Y., and Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. *Science* 291, 490–493. doi: 10.1126/science.291.5503.490

Tinoco, A. B., Nisembaum, L. G., de Pedro, N., Delgado, M. J., and Isorna, E. (2014). Leptin expression is rhythmic in brain and liver of goldfish (*Carassius auratus*). *Role Feed. Time. Gen. Comp. Endocrinol.* 204, 239–247. doi: 10.1016/j.ygcen.2014.06.006

Tsang, A. H., Barclay, J. L., and Oster, H. (2014). Interactions between endocrine and circadian systems. *J. Mol. Endocrinol.* 52, R1–R16. doi: 10.1530/JME-13-0118

Vatine, G., Vallone, D., Gothilf, Y., and Foulkes, N. S. (2011). It's time to swim! Zebrafish and the circadian clock. *FEBS Lett.* 585, 1485–1494. doi: 10.1016/j.febslet.2011.04.007

Velarde, E., Haque, R., Iuvone, P. M., Azpeleta, C., Alonso-Gómez, A. L., and Delgado, M. J. (2009). Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic expression of period and Cryptochrome transcripts in retina, liver, and gut. *J. Biol. Rhythms* 24, 104–113. doi: 10.1177/0748730408329901

Vera, L. M., de Pedro, N., Gómez-Milán, E., Delgado, M. J., Sánchez-Muros, M. J., Madrid, J. A., et al. (2007). Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. *Physiol. Behav.* 90, 518–524. doi: 10.1016/j.physbeh.2006.10.017

Vera, L. M., Negrini, P., Zagatti, C., Frigato, E., Sánchez-Vázquez, F. J., and Bertolucci, C. (2013). Light and feeding entrainment of the molecular circadian clock in a marine teleost (*Sparus aurata*). *Chronobiol. Int.* 30, 649–661. doi: 10.3109/07420528.2013.775143

Vivas, Y., Azpeleta, C., Feliciano, A., Velarde, E., Isorna, E., Delgado, M. J., et al. (2011). Time-dependent effects of leptin on food intake and locomotor activity in goldfish. *Peptides* 32, 989–995. doi: 10.1016/j.peptides.2011.01.028

Welsh, D. K., Takahashi, J. S., and Kay, S. A. (2010). Suprachiasmatic nucleus: cell autonomy and network properties. *Annu. Rev. Physiol.* 72, 551–577. doi: 10.1146/annurev-physiol-021909-135919

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Influencia del horario de alimentación sobre los receptores nucleares PPARα y REV-ERBα en el hipotálamo y el hígado de carpín

Introducción

Los receptores nucleares son una superfamilia de factores de transcripción que constituyen un factor esencial en la homeostasis energética de diferentes tejidos periféricos relacionados con el metabolismo. Esto es debido a que estos receptores nucleares intervienen en una gran cantidad de funciones, entre las que se encuentran la regulación de la expresión de genes involucrados en el metabolismo glucídico y lipídico, en el almacenamiento de lípidos, en la lipogénesis o en la β-oxidación de los ácidos grasos en el hígado de los mamíferos (Eckel-Mahan y Sassone-Corsi, 2013; Chen y Yang, 2014; Pawlak *et al.*, 2015; Vieira *et al.*, 2015). Asimismo, en la última década ha cobrado una gran importancia la hipótesis de que algunos de estos receptores nucleares como PPARs y REV-ERBs son factores de transcripción claves para la conexión entre el metabolismo y el sistema circadiano en los mamíferos, siendo por tanto elementos centrales para el mantenimiento de una óptima homeostasis temporal (Vieira *et al.*, 2015; Ribas-Latre y Eckel-Mahan, 2016; Albrecht y Ripperger, 2018).

Por lo tanto, nos propusimos investigar el posible efecto de un cambio en el horario de alimentación sobre la expresión de los receptores nucleares PPARα y REV-ERBα en el carpín, como modelo de pez teleósteo.

Material y Métodos

Para llevar a cabo este estudio se utilizaron individuos de carpín (*Carassius auratus*, *n* = 72) mantenidos con unas condiciones de fotoperiodo 12 horas de luz y 12 horas de oscuridad (12L:12D; encendido de las luces a las 08:00) y alimentación fija a las 10:00 (ZT2). Pasadas dos semanas bajo estas condiciones de aclimatación, se les sometió al protocolo experimental empleado en el Capítulo 2.2 (**Figura 18**).

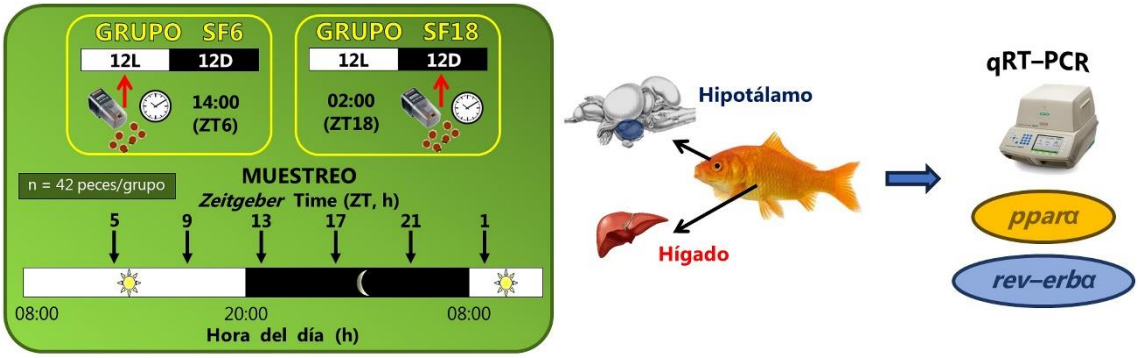


Figura 18. Esquema del diseño experimental. RT-qPCR, PCR cuantitativa en tiempo real; SF, *scheduled fed* o alimentación fija.

Brevemente, dos grupos de animales se mantuvieron con las mismas condiciones de fotoperiodo (12L:12D). A uno de ellos (n = 36) se le dio de comer en mitad del periodo diurno (14:00, ZT6, llamado SF6, *scheduled fed at ZT6*), mientras que al otro grupo (n = 36) se les proporcionó la comida en medio de la fase nocturna (02:00, ZT18, llamado SF18). Transcurridas tres semanas, los peces fueron muestreados cada 4 horas a lo largo de todo un ciclo de 24 horas (a ZT5, ZT9, ZT13, ZT17, ZT21 y ZT1). Los animales fueron sacrificados con una

sobredosis de anestésico (metanosulfonato de tricaína o MS-222, 0.28 g/l) y de cada animal se extrajeron rápidamente tanto el hipotálamo como el hígado para analizar la expresión génica de *pparα* y *rev-erbα* en ambos tejidos.

Resultados

Los resultados obtenidos en el hipotálamo muestran que sólo *rev-erbα* exhibe ritmos diarios significativos con las acrofases durante la noche (ZT18,2 en animales SF6 y ZT15,4 en animales SF18; **Figura 19**). Es decir, el cambio en el horario de alimentación de ZT6 a ZT18 sólo adelantó unas 3 h la acrofase de *rev-erbα* en el hipotálamo, mientras que reduce (aproximadamente 5 horas) la amplitud del ritmo diario de este factor de transcripción.

En el hígado, ambos receptores (PPARα y REV-ERBα) presentan ritmos diarios significativos tanto en los peces SF6 como en los SF18 (**Figura 19**). En los animales SF6, la acrofase de pparα ocurrió al inicio del día (ZT3,4), mientras que la de rev-erbα en mitad de la fase oscura (ZT19,0, similar a la del hipotálamo). Cuando el horario de la alimentación se desfasó desde ZT6 a ZT18, las acrofases de ambos genes también se desplazaron 12 horas (pasando de estar en ZT3,4 en SF6 a ZT15,8 en SF18 para pparα y de ZT19,0 a ZT7,4 para rev-erbα). Por su parte, las amplitudes no mostraron cambios sustanciales en los animales SF6.

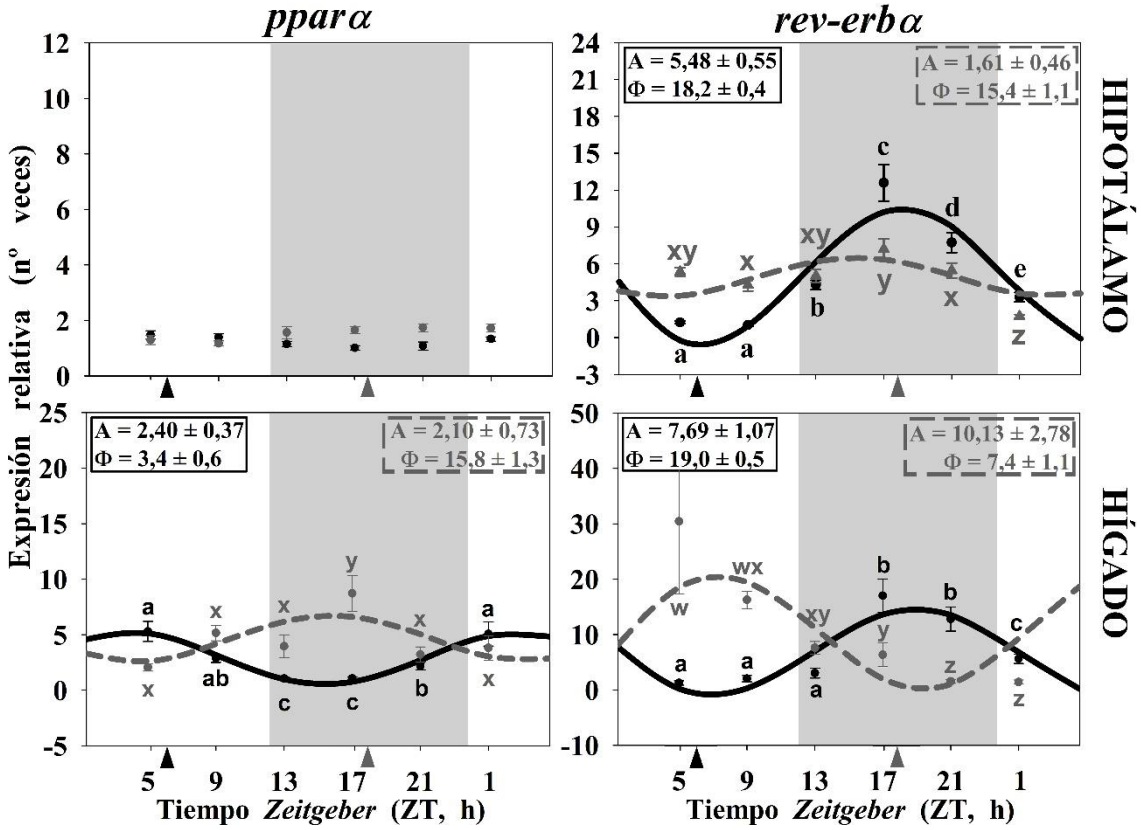


Figura 19. Perfil diario de expresión de *pparα* y *rev-erbα* en el hipotálamo (arriba) y el hígado (abajo) de los carpines SF6 (●) y SF18 (▲). El área gris indica el periodo de oscuridad, mientras que el momento de la alimentación viene definida por los triángulos en el eje X (negro, ZT6; gris, ZT18). Los datos obtenidos mediante RT-qPCR se muestran como la media ± SEM (*n* = 6) en unidades relativas (método del $2^{-\Delta\Delta Ct}$). Las diferentes letras (a-d en SF6 y w-z en SF18) indican diferencias significativas por ANOVA y Student-Newman-Keuls (SNK) *post hoc* test. Cuando el análisis Cosinor (*SE(A)/A* < 0.3) y el ANOVA resultaron significativos, se representaron las funciones sinusoidales de los ritmos con ondas continuas negras (SF6) o discontinuas grises (SF18). Las amplitudes (A, unidades relativas) y acrofases (ϕ, horas) se muestran en la parte superior de cada gráfica (a la izquierda en negro SF6 y a la derecha en gris SF18).


DISCUSIÓN

DISCUSIÓN

En la presente Discusión, se pretenden destacar las principales ideas sobre la organización temporal y el control metabólico y neuroendocrino de la homeostasis energética en el carpín derivadas de la visión general de los resultados obtenidos en los capítulos que constituyen esta Tesis Doctoral.

1 PAPEL DE LAS N–ACILETANOLAMINAS EN LA HOMEOSTASIS ENERGÉTICA

1.1 El sistema de las NAEs en el carpín

Con los resultados obtenidos en la presente Tesis Doctoral se ha podido caracterizar por primera vez en los peces teleósteos todos los componentes del sistema de las N-aciletanolaminas. Gracias a la técnica de espectrometría de masas acoplada a cromatografía líquida de ultra resolución (UPLC-MS, de sus siglas en inglés *ultra performance liquid cromatography – tandem mass spectrometer*), se ha podido demostrar la existencia de las tres principales NAEs (OEA, PEA y SEA), presentando todas ellas una amplia distribución tisular en el carpín, tal y como había sido demostrado previamente para la OEA en la misma especie (Tinoco *et al.*, 2014a). Así, se han conseguido obtener niveles detectables de todos los compuestos en dos tejidos cerebrales, como son el hipotálamo y el telencéfalo, y en tres tejidos gastrointestinales, como son el bulbo intestinal (homólogo del estómago en estos peces), el intestino anterior y el hígado. En estos mismos tejidos también se han detectado los niveles tisulares de los precursores de cada una de las NAEs (OEA, PEA y SEA), las denominadas NAPEs. Comparando los niveles en valor absoluto que hemos obtenido en los diferentes

tejidos del carpín con los descritos hasta la fecha en los mamíferos (Murillo-Rodriguez *et al.*, 2006; Fu *et al.*, 2007; Hansen y Diep, 2009; Guijarro *et al.*, 2010; Liedhegner *et al.*, 2014) y en los reptiles (Astarita *et al.*, 2006), tanto los tejidos centrales como los gastrointestinales presentan cantidades muy similares de las tres NAEs a las descritas en los mamíferos, estando en el orden de unos poco nanomoles por cada gramo de tejido. Estos hallazgos nos llevan a pensar que estos derivados lipídicos bioactivos se encuentran ampliamente extendidos y conservados a lo largo de la evolución de los vertebrados.

También han sido caracterizadas las enzimas de síntesis (NAPE-PLD) y de degradación (FAAH) en el carpín a nivel de expresión génica en ambos casos, así como también de actividad enzimática en el caso de la FAAH. En la presente Tesis Doctoral y gracias a las técnicas genómicas se ha conseguido obtener una secuencia parcial de la enzima de síntesis NAPE-PLD, siendo analizada su expresión en tejidos centrales (hipotálamo; datos no mostrados) y periféricos (bulbo intestinal, intestino anterior e hígado). Por otro lado, hay que tener en cuenta que para ambas rutas de síntesis y degradación existen vías alternativas que todavía no han sido estudiadas en el carpín. Así, en cuanto a la síntesis de NAEs a partir de las NAPEs pueden existir otras tres vías complementarias a la hidrólisis directa que ejerce la NAPE-PLD (Hussain *et al.*, 2017; Inoue *et al.*, 2017). Por su parte, la degradación de las NAEs se realiza principalmente por la enzima FAAH, pero en mamíferos se ha descrito la acción de otra enzima que tiene también alta afinidad por la OEA y sobre todo la PEA, que es la NAAA. Al igual que con las rutas alternativas de síntesis, esta otra enzima catabólica todavía no se ha caracterizado en el carpín, aunque parece ser que se ha perdido en el pez cebra (McPartland *et al.*, 2007).

De todos los receptores de las NAEs que se han descrito hasta la actualidad en los mamíferos, en la presente Tesis Doctoral nos hemos centrado en el estudio del PPARα, ya que es el que parece mediar los efectos de estas NAEs en la regulación de la homeostasis energética. En concreto, este receptor presenta una amplia distribución corporal en el carpín encontrándose sus tránscritos tanto en tejidos centrales (hipotálamo) como en tejidos periféricos (bulbo intestinal, intestino anterior e hígado). Como se comentará más adelante, este receptor nuclear podría estar implicado en el metabolismo lipídico y la modulación del sistema circadiano como en los mamíferos. Sin embargo, en relación con el resto de receptores a los que se pueden unir las NAEs (TRPV1, GPRs e incluso canales iónicos), actualmente se desconocen tanto sus distribuciones tisulares como las posibles implicaciones en las acciones fisiológicas de las NAEs a través de estos receptores en el carpín.

1.2 Las NAEs como señales postprandiales de saciedad

La movilización de las NAEs a nivel intestinal que se produce en respuesta a la alimentación, tal y como se comentó en la Introducción, sugiere que estas señales lipídicas podrían intervenir en la regulación de la ingesta como señales periféricas de saciedad tanto en mamíferos (Rodríguez de Fonseca *et al.*, 2001; Fu *et al.*, 2007) como en reptiles (Astarita *et al.*, 2006). Los resultados obtenidos en el carpín en la presente Tesis Doctoral en relación con el claro aumento postprandial (1 hora) comparado con 25 horas de ayuno también apoyan esta idea. Mientras que para la OEA ya había sido descrito un resultado similar en la misma especie por nuestro grupo de investigación (Tinoco *et al.*, 2014a), es la primera vez que se han demostrado cambios periprandiales de PEA y SEA en tejidos gastrointestinales de los peces. En los mamíferos, el aumento postprandial de las NAEs se ha relacionado con cambios en paralelo en el contenido gastrointestinal de las NAPEs (Petersen *et al.*, 2006; Fu *et al.*, 2007; Gillum *et al.*, 2008; Schwartz *et al.*, 2008; Bowen *et al.*, 2017) y/o cambios en la actividad de los enzimas NAPE-PLD y FAAH (Fu *et al.*, 2007; Schwartz *et al.*, 2008; Bowen *et al.*, 2017). Sin embargo, nuestros resultados parecen no corroborarlo en los peces, ya que ni las NAPEs ni la expresión génica de ninguna de los dos enzimas se ven modificadas por el estado nutricional de los peces. Podemos plantear algunas hipótesis que podrían justificar nuestros resultados, como la existencia de vías alternativas de síntesis de NAEs que no implican a la NAPE-PLD como la enzima principal o una mayor contribución de otras amidasas en el catabolismo de las NAEs (Hussain *et al.*, 2017; Inoue *et al.*, 2017; Lin *et al.*, 2018). El hecho de que los cambios tan drásticos observados en los tejidos gastrointestinales no se observaran en el hipotálamo y en el telencéfalo, sugieren en primer lugar que existe una marcada regulación tejido-dependiente de las NAEs por la ingestión de alimentos en el carpín y, en segundo lugar, que las NAEs parecen estar actuando como señales periféricas de saciedad a corto plazo en los peces teleósteos.

Trabajos previos de nuestro laboratorio confirmaron este papel de la OEA como señal anorética tras su administración IP en el carpín (Tinoco *et al.*, 2014a), corroborando estudios previos en los reptiles (Astarita *et al.*, 2006) y en los mamíferos (Rodríguez de Fonseca *et al.*, 2001; Fu *et al.*, 2003, 2005; Serrano *et al.*, 2011). Con el fin de confirmar este posible efecto anorético para otras NAEs como la PEA y la SEA se llevó a cabo una aproximación farmacológica de administración IP de forma aguda en el carpín. En ambos casos se observó una reducción de la ingesta, aunque solo fue estadísticamente significativa en el caso de la PEA. Aunque la dosis ensayada de SEA (20 mg/kg, datos no mostrados) es similar a la utilizada en el único estudio realizado en los mamíferos (Terrazzino *et al.*, 2004), en concreto en ratones

(25 mg/kg); sería interesante ensayar otras dosis, quizás más elevadas para poder confirmar un claro efecto anorético de la SEA en los peces. En relación al efecto anorético de la PEA en el carpín, nuestros resultados corroboran datos previos en ratas tras tratamientos tanto agudos (Rodríguez de Fonseca *et al.*, 2001) como crónicos (Mattace Raso *et al.*, 2014a).

La regulación homeostática de la ingesta en los peces es la respuesta de la integración hipotalámica de las señales metabólicas y endocrinas, las cuales provocan a su vez cambios en la expresión de distintos neuropéptidos tanto orexigénicos como anoréticos (Delgado *et al.*, 2017; Soengas *et al.*, 2018). Por ello, para profundizar en los mecanismos que pueden estar mediando la reducción de la ingesta en el carpín por parte de las NAEs, se analizó la expresión génica de distintas señales centrales y periféricas involucradas en la regulación de la alimentación en los peces. Mientras que el efecto anorético de la OEA parece no estar mediado por cambios hipotalámicos de NPY y orexina (Tinoco *et al.*, 2014a), en el caso de la PEA se ha observado una drástica caída en los niveles de expresión hipotalámica de *npy*. Estos resultados son comparables con las acciones que generan otras señales anoréticas sobre la expresión del *npy* hipotalámico, como pueden ser un incremento en los niveles de nutrientes o la secreción de hormonas anoréticas (Delgado *et al.*, 2017; Soengas *et al.*, 2018). En ratas ovariectomizadas, un tratamiento agudo con PEA provocó un aumento del neuropéptido anorético POMC (Mattace Raso *et al.*, 2014b), mientras que en el carpín no se observaron modificaciones significativas en ninguno de los dos neuropéptidos anoréticos estudiados en el hipotálamo (*pomc* y *cartpt*). Estas discrepancias pueden ser debidas a diferentes causas: diferencias interespecíficas (peces *versus* mamíferos), duración del tratamiento (10 días en los peces *versus* 5 semanas en los mamíferos) o diferentes aproximaciones experimentales (carpín de fenotipo salvaje *versus* modelo de obesidad inducido por ovariectomía en rata). Entre las posibles señales neuroendocrinas de carácter periférico que podrían estar implicadas en los efectos de las NAEs en los peces, se han estudiado la ghrelina, la colecistocinina y la leptina. Mientras que la disminución de la ingesta producida por la OEA parece estar mediada por una reducción en la ghrelina a nivel intestinal tanto en mamíferos (Cani *et al.*, 2004; Serrano *et al.*, 2011) como en peces (Tinoco *et al.*, 2014a), el tratamiento agudo con PEA no provocó ningún cambio en los niveles de esta hormona en el bulbo intestinal del carpín (datos no mostrados), sugiriendo que puede existir una regulación diferencial de la expresión intestinal de *ghrelina* en el carpín en función de la NAE estudiada. En cuanto a la colecistocinina, parece ser que esta hormona no está implicada en los efectos anoréticos de la OEA en mamíferos (Proulx *et al.*, 2005) y peces (Tinoco *et al.*, 2014a), ni de la PEA en los peces teleósteos (datos no mostrados). Por último, se postula que el sistema de la leptina no parece intervenir en la acción anorética

de la OEA tanto en mamíferos como en peces, ya que en ratas Zucker obesas (sin receptores funcionales para la leptina) el tratamiento con OEA sigue reduciendo la ingesta (Fu *et al.*, 2005); y en el carpín, la OEA no indujo ningún cambio en la expresión de la *leptina* a nivel hepático e hipotalámico (Tinoco *et al.*, 2014a). Por otro lado, un tratamiento agudo de PEA provocó un marcado aumento en los niveles de expresión de la *leptina* hepática, lo que podría implicar un aumento en los niveles circulantes de leptina que, tras su integración hipotalámica, provocaría la disminución observada en los niveles de *npy*, tal y como se ha demostrado previamente en el carpín (Volkoff *et al.*, 2003) y la trucha arcoíris (Murashita *et al.*, 2008; Aguilar *et al.*, 2011). Estos datos concuerdan con lo observado en los mamíferos, ya que la PEA provocó un aumento de la señalización hipotalámica generada por la leptina (Mattace Raso *et al.*, 2014a), a pesar de que no se encontraron modificaciones en los niveles circulantes de esta hormona (Mattace Raso *et al.*, 2014b). Por último, resulta interesante tener en cuenta que estudios muy recientes en mamíferos apuntan a que el papel que ejercen las NAEs sobre la regulación de la ingesta parece ser más bien a través de mecanismos hedónicos en vez de homeostáticos (Monteleone *et al.*, 2016). Esta posible regulación hedónica podría ser la respuesta a las discrepancias encontradas al observar los efectos anorexigénicos provocados por las NAEs y los causados por otras condiciones anoréticas entre los peces y los mamíferos. Por ello, se requiere la realización de nuevos diseños experimentales que permitan abordar y clarificar el papel de las NAEs en los circuitos implicados en la regulación hedónica en los peces teleósteos.

Cabe destacar también los resultados de la presente Tesis Doctoral relacionados con la reducción de peso corporal observada tras un tratamiento crónico durante 10 días con OEA o PEA. Dichos resultados están de acuerdo con estudios previos realizados en roedores que han mostrado, junto a la reducción de la ingesta, una reducción del peso corporal tras el tratamiento crónico con OEA (Rodríguez de Fonseca *et al.*, 2001; Guzmán *et al.*, 2004; Fu *et al.*, 2005; Serrano *et al.*, 2008) y con PEA (Mattace Raso *et al.*, 2014b). Otro de los parámetros que está relacionado con la homeostasis energética de los animales es la actividad locomotora. Así, tratamientos agudos con OEA provocan que las ratas se muevan significativamente menos, manteniéndose un mayor tiempo en inactividad y presentando menos periodos de deambulación en la prueba del campo abierto (Rodríguez de Fonseca *et al.*, 2001; Proulx *et al.*, 2005). Por su parte, la PEA también genera una reducción en la actividad locomotora de los ratones tratados con esta NAE en la prueba de campo abierto (Zambrana-Infantes *et al.*, 2018). Nuestros resultados en el carpín confirman que el tratamiento crónico con OEA y PEA reducen la actividad locomotora, de forma similar a lo descrito previamente en mamíferos, así como

tras una administración aguda de OEA en el carpín (Tinoco *et al.*, 2014a).A pesar de experimentar simultáneamente estas disminuciones en la actividad locomotora y en la ingesta, parece ser que ambos procesos son independientes y ninguno de los dos son consecuencia del otro, como se ha sugerido en rata (Rodríguez de Fonseca *et al.*, 2001). En los peces, esta regulación independiente de la actividad locomotora y de la actividad alimentaria se encuentra respaldada por diversos trabajos realizados en nuestra especie de estudio, el carpín (Sánchez-Vázquez *et al.*, 1996; Azpeleta *et al.*, 2010; Vivas *et al.*, 2011). Por otra parte, el comportamiento alimentario suele incluir tres fases secuenciales en los peces: una fase de excitación o alerta encaminada a la búsqueda de la comida, seguida de una fase de apetito en la que se localiza el alimento y una fase final consumatoria que consiste en la propia ingestión de los alimentos (Lamb, 2001; Azpeleta *et al.*, 2010). Teniendo en cuenta toda esta información, resulta muy interesante nuestro resultado tras el tratamiento crónico con PEA que demuestra tanto una disminución de la fase consumatoria de la alimentación como una importante reducción de la actividad anticipatoria al alimento, que está relacionada más directamente con las primeras fases de alerta y localización de la comida y, por tanto, con aspectos motivacionales de la alimentación.

1.3 Regulación del metabolismo hepático por las NAEs

Este capítulo de la presente Tesis Doctoral aporta los primeros datos sobre un posible papel de la OEA y la PEA en el metabolismo de lípidos y de glúcidos a nivel hepático en los peces teleósteos.

Respecto al metabolismo lipídico, ambas NAEs estudiadas presentan un efecto lipogénico en el hígado del carpín, caracterizado por el aumento de la actividad enzimática de ACLY por acción de la OEA y de FAS (ácido graso sintasa) por parte de la PEA, dos enzimas implicadas en la ruta de biosíntesis de ácidos grasos. Cabría esperar que si la capacidad lipogénica hepática está aumentada por el tratamiento con estos derivados lipídicos, el potencial lipolítico se viese disminuido o, al menos, no modificado. Esta idea se corrobora efectivamente para las dos NAEs. En el caso de la OEA, se observa una marcada reducción de la actividad enzimática de la CPT-1, sugiriendo una reducción en la β-oxidación mitocondrial de ácidos grasos, mientras que la PEA no generó ninguna modificación de la lipólisis hepática. Estos cambios en los potenciales lipogénico y lipolítico podrían estar asociados, al menos parcialmente, con el aumento de la expresión génica de *bmal1a* que encontramos paralelamente en el hígado tras el tratamiento con OEA y PEA. De hecho, en mamíferos se ha demostrado también que este gen está implicado en la regulación lipídica, aumentando la lipogénesis y disminuyendo la lipólisis tanto en hepatocitos de ratón (Zhang D *et al.*, 2014) como en adipocitos de rata

(Shimba *et al.*, 2005, 2011). En los mamíferos se ha demostrado un claro efecto lipolítico de la OEA en el tejido adiposo (Decara *et al.*, 2012; Suárez *et al.*, 2014), mientras que su papel en el hígado no está tan claro. Algunos estudios sugieren un aumento del potencial lipolítico (Guzmán *et al.*, 2004; Fu *et al.*, 2005), mientras que en otros parece reducirse (Serrano *et al.*, 2008; Thabuis *et al.*, 2011), resultado este último que coincidiría con nuestros resultados. Parece por tanto, que los efectos de la OEA en la regulación metabólica de lípidos podría ser dependiente del tejido estudiado. Además, no podemos descartar que en el carpín no esté teniendo lugar un aumento de la lipólisis en otros tejidos distintos al hígado, como sugiere el hecho de que se reduzcan los niveles plasmáticos de triglicéridos, y aumenten los de ácidos grasos, resultado similar al descrito en roedores (Fu *et al.*, 2005; Yang *et al.*, 2007; Thabuis *et al.*, 2011). Por último, querríamos resaltar que en la mayoría de estudios realizados en los mamíferos únicamente investigan el papel de la OEA (no existiendo apenas datos de la PEA), y cuantifican expresión génica de enzimas o metabolitos plasmáticos, pero no la actividad enzimática directamente, tal y como hemos abordado en nuestros estudios, todo lo que hace difícil una posible analogía entre los resultados publicados en mamíferos y los obtenidos en peces en la presente Tesis Doctoral.

Entre los efectos de la OEA y la PEA en el metabolismo glucídico cabe destacar una importante reducción en el potencial gluconeogénico, a partir de la drástica disminución que se observa en la actividad hepática de la PEPCK (enzima principal de la gluconeogénesis o síntesis *de novo* de la glucosa) en ambos casos. Esta reducción en la actividad de la PEPCK podría estar asociada, al menos en parte, con el incremento observado en la expresión de *bmal1a*, si tenemos en cuenta que en los mamíferos la sobreexpresión de este gen induce una reducción en el ARNm de la *pepck* en adipocitos (Shimba *et al.*, 2005). Por otro lado, el aumento en la actividad GPasa (glucógeno fosforilasa, enzima principal de la degradación de glucógeno) tras el tratamiento con OEA y PEA sugiere que ambas NAES también incrementan el potencial glucogenolítico en el carpín. A pesar de estos efectos similares de la OEA y la PEA, nuestros resultados sugieren una diferente regulación por ambas NAEs en lo que se refiere a la capacidad hepática para usar la glucosa. Mientras que la OEA parece reducir la fosforilación de la glucosa y su uso, tal y como indica la reducción en la actividad de las enzimas HK y GK; en el caso de la PEA, la actividad de estas dos enzimas no fue modificada, lo que junto a una mayor actividad enzimática de la PFK (fosfofructoquinasa, una de las enzimas clave de la glucólisis), sugiere una elevada capacidad para utilizar la glucosa hepática. Por el momento desconocemos qué mecanismos desencadenan este diferente efecto de las dos NAEs estudiadas sobre la capacidad de uso de la glucosa a nivel hepático. El efecto de la OEA podría

ser comparable con los resultados descritos en rata, donde esta NAE reduce la oxidación de la glucosa en el hígado (Thabuis *et al.*, 2011), mientras que en mamíferos hasta el momento no hay ningún dato de la posible relación de la PEA y el metabolismo glucídico.

Estos resultados ponen de manifiesto por primera vez en los peces una posible interacción entre las NAEs-sistema circadiano-metabolismo muy interesante, y que está en línea con lo descrito en mamíferos sobre la interacción sistema circadiano-metabolismo, teniendo como posibles nexos elementos de la maquinaria molecular del oscilador como PPAR y BMAL (**Figura 20**; Charoensuksai y Xu, 2010; Chen y Yang, 2014; Ribas-Latre y Eckel-Mahan, 2016).

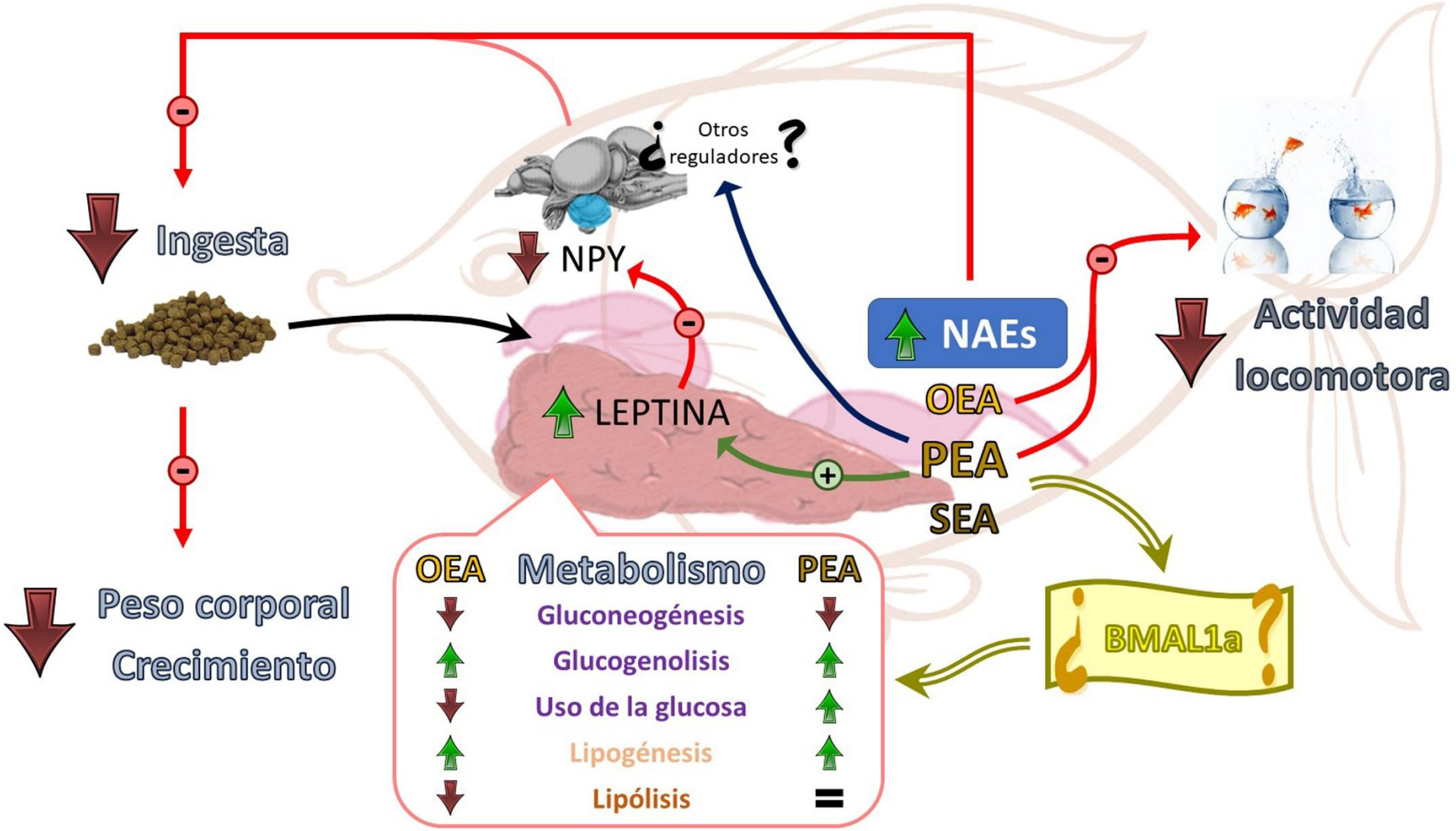


Figura 20. Representación esquemática de los principales efectos de las NAEs en la homeostasis energética del carpín. NAEs, N-aciletanolaminas; NPY, neuropéptido Y; OEA, oleiletanolamida; PEA, palmitoiletanolamida; SEA, estearoiletanolamida. ⊕, estimulación; ⊖, inhibición.

2 RITMICIDAD DE SEÑALES RELACIONADAS CON LA HOMEOSTASIS ENERGÉTICA

El sistema circadiano de los vertebrados es el responsable de la regulación de la ritmicidad de una gran cantidad de funciones fisiológicas, metabólicas y comportamentales, de tal forma que adecúa la homeostasis a los cambios diarios ambientales para un correcto funcionamiento del organismo (Albrecht y Ripperger, 2018). Por este motivo, en la presente Tesis Doctoral nos hemos propuesto profundizar en el conocimiento del sistema circadiano y la homeostasis energética en el carpín como modelo animal de teleósteo.

2.1 Sincronización de los osciladores por los *zeitgeber*s externos: ciclos luz-oscuridad y alimentación-ayuno

Tal y como se comentó en la Introducción de la presente Tesis Doctoral, el sistema circadiano de los peces es una red de osciladores sin una jerarquización como ocurre en los mamíferos, lo que podría conferirles una mayor flexibilidad a la hora de su sincronización. La actividad locomotora diaria es una de las salidas circadianas más robustas que posee el sistema circadiano, pudiendo ser encarrilada por cambios en los *zeitgeber*s tanto en mamíferos (Refinetti, 2015) como en los peces teleósteos (Aranda *et al.*, 2001; López-Olmeda *et al.*, 2009; Sanchez y Sanchez-Vazquez, 2009). En relación con este hecho, se ha considerado que la especie de nuestro estudio, el carpín, presenta un comportamiento locomotor muy adaptable adecuándose en todo momento a la disponibilidad del alimento (Aranda *et al.*, 2001; Feliciano *et al.*, 2011; Isorna *et al.*, 2017). Con los resultados de la presente Tesis Doctoral se pueden confirmar los resultados previamente descritos ya que bajo unas condiciones de fotoperiodo 12L:12D, observamos que los peces que obtuvieron la comida en mitad del periodo diurno concentraron toda su actividad locomotora anticipatoria al alimento (FAA) en dicho periodo, mientras que los animales alimentados en mitad de la noche presentaban su FAA durante el periodo nocturno. Estos resultados también se obtuvieron cuando se analizó la actividad locomotora relacionada con la alimentación registrada exclusivamente en la zona alrededor del comedero automático. Sin embargo, los peces sincronizaron la mayoría de su actividad locomotora general al periodo de luz o fotofase, independientemente del momento de la alimentación. Es decir, los animales que comen durante la fotofase son claramente diurnos, mientras que los peces que comieron durante la escotofase (fase nocturna) no son nocturnos. Esto no ocurre igual en todas las especies de peces ya que Vera y colaboradores (2013) describieron que en la dorada tanto si se proporciona el alimento durante el día como si se les alimenta por la noche, los peces adecúan en torno al 90% de toda su actividad a dichos periodos de alimentación. Sin embargo, hay que

tener en cuenta que estos autores no cuantificaron de forma separada la actividad general y la ligada a la alimentación, como se ha realizado en la presente Tesis Doctoral, por lo que no sería posible discriminar cuánta cantidad de esa actividad está generada por cada una de las dos actividades aquí analizadas, pudiendo en tal caso llegar a la misma conclusión que nosotros en el carpín. Nuestros resultados también apoyarían la existencia de dos osciladores LEO y FEO separados, uno sincronizado por el fotoperiodo y otro por el alimento (Gillman *et al.*, 2019), lo que sugiere que los distintos tipos de actividad locomotora no están controlados por las mismas estructuras, tal y como ocurre en los mamíferos. Es de sobra conocido que el hipotálamo es el oscilador LEO, pero hasta el momento la localización del oscilador FEO sigue siendo un misterio (López-Olmeda, 2017), aunque se ha buscado en distintos tejidos. Por una parte, y aunque los datos presentes podrían indicar que el principal FEO es el hígado, en el carpín se ha descartado esta hipótesis debido al hecho de que, en condiciones de luz constante, este tejido no presenta ritmos de FAA, pero sí de los genes que componen el oscilador hepático (Feliciano *et al.*, 2011). Por otra parte, tampoco parece ser que la actividad locomotora en el carpín dependa del ritmo de cortisol debido a que en situaciones en las que los peces no presentan ritmos de esta hormona se siguen manteniendo los ritmos diarios de actividad locomotora (datos de la presente Tesis Doctoral).

Todos los resultados mostrados en la presente Tesis Doctoral en los diferentes experimentos (capítulo 1 – subcapítulo 1.3 y capítulo 2 – subcapítulos 2.1 y 2.2) evidencian que cuando los animales son alimentados por el día, los genes *period* (mitad negativa del bucle principal) se encuentran en antifase con los genes *clock1a*/*bmal1a* (mitad positiva) tanto en el hipotálamo como en el hígado, excepto en el caso de *per2a* que se discutirá más adelante. Además, todos los genes reloj estudiados exhibieron sus máximos de expresión o acrofases en momentos similares haciendo que los osciladores tanto del tejido central como del periférico estén en fase entre sí fluctuando a la vez, tal y como se puede observar en la **Tabla 3**.

Por un lado, los genes *per1a* y *per1b* presentan sus acrofases alrededor del momento de la transición oscuridad-luz en ambos tejidos, independientemente de una alimentación fija al inicio (ZT2) o a mitad (ZT6) del periodo diurno. Estos datos apoyan la hipótesis que afirma que estos genes *per1* anticipan la llegada de la luz en los peces teleósteos bajo condiciones de fotoperiodo 12L:12D con una alimentación durante el periodo diurno (Isorna *et al.*, 2017). A pesar de ello, ambos genes en el hígado tienden a enlazarse un poco más con la alimentación, tal y como se puede observar en sus acrofases desplazadas a momentos iniciales del día cuando los peces fueron alimentados a ZT6, respecto a momentos finales de la noche cuando lo fueron a ZT2. De hecho, parece ser que *per1b* no está tan estrictamente vinculado a la

Tabla 3. Acrofases de los genes reloj y receptores nucleares en el hipotálamo y el hígado obtenidas en los distintos experimentos de la presente Tesis Doctoral en carpines mantenidos en 12L:12D con distintos horarios de alimentación (ZT2, ZT6, ZT18 y aleatorio).

	Hipotálamo				Hígado			
	ZT2	ZT6	ZT18	Aleatoria	ZT2	ZT6	ZT18	Aleatoria
per1a	22,3	22,7	17,3	21,8	22,7	0,7	13,1	20,0
per1b	23,1	1,3	21,1	23,3	22,5	0,9	11,6	20,4
per2a	4,3	7,6	-	3,8	-	-	-	-
per3	1,0	4,1	19,7	1,0	0,5	3,5	13,8	22,3
clock1a	-	14,3	-	-	5,4	10,0	23,6	8,6
bmal1a	8,8	11,3	6,6	8,3	7,7	9,0	0,3	7,6
pparα	-	-	-	-	0,5	3,4	15,8	22,0
rev-erbα	18,6	18,2	15,4	17,6	17,1	19,0	7,4	16,9

llegada de la luz como *per1a*, ya que presenta mayores modificaciones moviéndose su acrofase en función de la hora de la alimentación. Por su parte, el gen reloj *per3* parece estar más controlado por la alimentación tanto a nivel hipotalámico como hepático, ya que su acrofase cambia según varía el horario de alimentación. Esto apoya hipótesis previas que también vinculaban este gen *per3* a la alimentación en estos dos tejidos, al igual que en el techo óptico del carpín (Feliciano *et al.*, 2011; Nisembaum *et al.*, 2012). Cabe destacar que, cuando la alimentación fue durante el periodo nocturno, la sensibilidad de los osciladores parece ser dependiente del tejido. Así, en el hipotálamo del carpín parece que se produce una desregulación de las acrofases de los genes *period* respecto a lo que ocurría cuando los peces eran alimentados por el día, ya que los tres genes reloj tienen su acrofase en torno a la mitad de la escotofase. En cambio, las acrofases de estos genes en el hígado se mueven tal y como lo hace el horario de la alimentación. Otro resultado interesante es como parece afectar la alimentación aleatoria según el tejido. En el hipotálamo, la alimentación aleatoria (con la última toma a ZT2) no modifica las acrofases de los genes *per*, mientras que en el hígado adelanta las acrofases 2 horas en los tres casos. Estos resultados equivalen a lo descrito previamente en el hígado del carpín bajo condiciones de luz constante en el que una sola toma de alimentación es capaz de resetear el oscilador hepático (Feliciano *et al.*, 2011). En nuestro caso, los animales se encontraban bajo un fotoperiodo de 12L:12D y una sola toma de alimento empieza a resetear el reloj hepático para volver a estar en fase con el hipotalámico

aunque no completamente, lo que sugiere que la comida es un *input* muy fuerte para el hígado del carpín.

Por su parte, los genes *clock1a* y *bmal1a* presentan sus acrofases principalmente reguladas por el horario de la alimentación, tanto a nivel hipotalámico como a nivel hepático. De hecho, ambos genes presentan sus máximos de expresión unas 5-6 horas después del momento de la alimentación en ambos tejidos, a excepción del hipotálamo con alimentación nocturna, lo que parece ir en concordancia con los datos discutidos previamente de los genes *per* en los que también existía una desregulación del oscilador hipotalámico bajo dichas condiciones. Los datos obtenidos en el hipotálamo del carpín concuerdan con trabajos previos en el cerebro de la dorada (Vera *et al.*, 2013) y de la tilapia del Nilo (*Oreochromis niloticus*; (Costa *et al.*, 2016), aunque no así para el hígado, ya que ambos trabajos describieron unos cambios de solamente unas 6-8 horas en las acrofases de *clock1a* y *bmal1a* con inversiones de 12 horas del horario de alimentación, mientras que nuestros resultados muestran una inversión también de 12 horas en todos los genes reloj en el hígado del carpín.

Cuando el ciclo luz-oscuridad fue eliminado y los peces se mantuvieron en oscuridad constante, el oscilador hipotalámico se alteró de tal forma que los ritmos diarios de expresión de todos los genes reloj se perdieron haciendo un oscilador no funcional, aun a pesar de mantener fija la alimentación al inicio del supuesto periodo diurno. Este fue un resultado inesperado ya que trabajos previos de nuestro laboratorio habían descrito que la alimentación es capaz de sincronizar los ritmos diarios de expresión de los genes reloj bajo un régimen de luz constante, tanto en el techo óptico como en el hipotálamo (Feliciano *et al.*, 2011). Una posible explicación recaería en los genes inducibles por la luz *per2a* y *cry1a*, ambos considerados piezas clave para la sincronización fótica de los LEO en los peces teleósteos (Vatine *et al.*, 2011; Nisembaum *et al.*, 2012; Ben-Moshe *et al.*, 2014; Moore y Whitmore, 2014; Isorna *et al.*, 2017). Esta sincronización la llevan a cabo gracias a que en sus promotores presentan tanto cajas E (reconocidas por factores de transcripción) como cajas D (encargadas de la recepción de la entrada de la luz), ambas conservadas a lo largo de la evolución (Vatine *et al.*, 2009). De ahí que, en nuestros resultados bajo una oscuridad constante, *per2a* no pudo ser activado por la luz y con ello tampoco regular la maquinaria del reloj circadiano, convirtiendo al hipotálamo en un oscilador no rítmico en estas condiciones. Por su parte, en los resultados obtenidos por Feliciano y colaboradores (2011) los genes reloj presentan ritmos diarios de expresión posiblemente debido al *masking* producido por la constante activación de *per2a* en condiciones de luz constante. Sin embargo, el reloj hepático apenas sufrió modificaciones cuando se mantuvo a los peces en oscuridad constante, conservándose prácticamente intactos

los ritmos diarios de expresión de los genes reloj. Nuestros datos concuerdan con trabajos previos de nuestro laboratorio en los que una alimentación bajo un fotoperiodo de luz constante era capaz de sincronizar los relojes endógenos de tejidos periféricos como el hígado o el intestino (Velarde *et al.*, 2009; Feliciano *et al.*, 2011; Nisembaum *et al.*, 2012). De hecho, en el hígado del carpín, *per2a* carece por completo de ritmos diarios de expresión sugiriendo que este tejido no es fotosensible en nuestra especie a diferencia del pez cebra (Vatine *et al.*, 2011; Ben-Moshe *et al.*, 2014). Estos resultados en los que, a pesar de no tener ritmicidad *per2a*, los osciladores periféricos (como el hígado o el intestino) siguen siendo rítmicos en el carpín nos hacen pensar que este gen reloj no es tan indispensable para la generación y mantenimiento de los ritmos circadianos en los peces teleósteos como se sugiere en los mamíferos (Kim *et al.*, 2018).

Por último, en el tejido interrenal las acrofases de los genes *per1* y *clock1a*/*bmal1a* se encuentran totalmente en antifase cuando los peces fueron alimentados en mitad de la fotofase, confirmando la existencia de un reloj endógeno funcional en este tejido, tal y como está descrito en la glándula adrenal de los mamíferos (Son *et al.*, 2008; Kwon *et al.*, 2011). A pesar de ello, cambios en los sincronizadores externos parecen afectarle de forma diferente respecto al hipotálamo y al hígado. Así, las acrofases de los genes *per1* sufren una inversión de 12 horas cuando la alimentación se desplazó desde el mitad del día a la mitad de la noche, al igual que ocurría en el hígado. Esto puede ser debido a que el momento de la comida parece jugar un papel importante en su sincronización, como se ha descrito previamente en otros tejidos periféricos tanto de mamíferos (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Schibler *et al.*, 2003; Kornmann *et al.*, 2007; Mukherji *et al.*, 2015) como de peces (Cavallari *et al.* 2011; Feliciano *et al.* 2011; Nisembaum *et al.* 2012; Tinoco *et al.* 2014b). En cuanto a los genes *clock1a* y *bmal1a*, cuando la alimentación fue nocturna, *clock1a* dejó de estar en antifase con los genes *per* ya que se adelantó solamente 4 h, mientras que *bmal1a* perdió su ritmicidad, lo que nos hace cuestionarnos la funcionalidad del reloj en este tejido bajo condiciones de desfase alimentario, idea que se refuerza con la pérdida del ritmo diario de cortisol circulante. Es decir, un cambio en el horario de la alimentación puede considerarse como un factor estresante en el carpín, dando lugar a que la homeostasis temporal se encuentre alterada.

Los resultados obtenidos en la presente Tesis Doctoral apoyan la idea de que el hipotálamo se encuentra principalmente sincronizado y controlado por el ciclo luz-oscuridad actuando como un clásico oscilador LEO, como se ha descrito previamente en los mamíferos (Mendoza y Challet, 2009) y en peces (López-Olmeda *et al.*, 2010; Vera *et al.*, 2013; Tinoco *et al.*, 2014b). Por su parte también apoyan la hipótesis de que el horario de alimentación parece

ser el sincronizador más potente para el hígado del carpín, siendo en este caso un clásico oscilador FEO, confirmando previos resultados de nuestro grupo de investigación (Feliciano *et al.*, 2011) y al igual que ocurre en los mamíferos (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Kornmann *et al.*, 2007). En el caso del tejido interrenal no tenemos datos suficientes para determinar si posee un carácter de LEO o de FEO. Un dato a tener en cuenta es que las amplitudes de los genes reloj en todos los tejidos se vieron claramente reducidas cuando alguno de los dos principales *zeitgeber*s (ciclos luz-oscuridad y alimentación-ayuno) se eliminaron e incluso en el hipotálamo y en el tejido interrenal cuando el alimento se suministró por la noche, sugiriendo que ambas señales externas trabajan juntas para sostener el mecanismo molecular (Nisembaum *et al.*, 2012; Sánchez-Bretaño *et al.*, 2015a).

2.2 Ritmos de NAEs y receptores nucleares

Los resultados obtenidos en la presente Tesis Doctoral muestran que el contenido de las NAEs (OEA, PEA y SEA) no presenta variaciones rítmicas diarias a nivel central (hipotálamo y telencéfalo), lo que coincide con la ausencia de oscilaciones diarias en el contenido de OEA y PEA en el hipotálamo de ratón (Liedhegner *et al.*, 2014) y de OEA en diferentes regiones cerebrales de rata (Guijarro *et al.*, 2010). Por el contrario, Murillo-Rodriguez y colaboradores (2006) detectaron variaciones diarias de OEA y PEA en el hipotálamo, el hipocampo y el puente troncoencefálico de rata. A la vista de estos resultados y teniendo en cuenta que no son muchos los estudios sobre la ritmicidad de las NAEs en el cerebro, se puede sugerir que los cambios diarios en los niveles cerebrales de estos derivados lipídicos son específicos de la región neural analizada, tal y como se había sugerido previamente en los mamíferos (Liedhegner *et al.*, 2014). No obstante, no se puede descartar que las NAEs jueguen otros papeles fisiológicos muy importantes a nivel cerebral tal y como ocurre en los mamíferos (D'Agostino *et al.*, 2009; Mattace Raso *et al.*, 2014a; Petrosino y Di Marzo, 2017; Holubiec *et al.*, 2018; Tsuboi *et al.*, 2018), un tema de gran interés que aún no se ha investigado en los peces. Por el contrario, estas NAEs parecen seguir un patrón rítmico diario presentando variaciones a lo largo del día en sus contenidos gastrointestinales (bulbo intestinal, intestino anterior e hígado), con un incremento muy pronunciado y rápido solamente una hora después de la alimentación. Esto nos sugiere que las NAEs no son señales rítmicas *per se* sino que sus niveles en los tejidos gastrointestinales dependen de la alimentación. De hecho, si se analizan los perfiles diarios sin el muestreo realizado después de comer (ZT3), se observa que no hay ritmos, indicando que la alimentación es la principal señal responsable de la ritmicidad diaria de las NAEs en los tejidos gastrointestinales del carpín. Por lo tanto, las NAEs serian

potencialmente buenas candidatas a ser señales postprandiales locales que pudiesen sincronizar los FEOs como el hígado.

Debido a que las NAEs pueden considerarse señales rítmicas diarias y que su principal receptor, PPARα, también presenta ritmicidad, en la presente Tesis Doctoral hemos analizado cómo pueden interaccionar las NAEs con los genes reloj que conforman los osciladores endógenos circadianos. Así, hemos podido observar que los tratamientos tanto agudo como crónico con PEA provocan cambios en la expresión de algunos genes reloj en el hígado del carpín. En concreto, el tratamiento agudo de PEA causó un aumento de la expresión de *per1a* y una disminución en la de *per3*, mientras que el tratamiento crónico generó un aumento en la expresión de los genes *per1a*, *clock1a* y *bmal1a*. Estas modificaciones ocasionadas en el reloj hepático podrían ser las causantes de los cambios en la homeostasis energética provocados por la PEA en este teleósteo. Por ejemplo, el metabolismo glucídico en el hígado de los mamíferos es un claro ejemplo de la intrincada conexión que existe entre la regulación circadiana de la maquinaria del reloj hepático con el metabolismo periférico (Peng *et al.*, 2019; Reinke y Asher, 2019), de tal forma que modificaciones en los genes que conforman los osciladores endógenos llevan asociados graves desórdenes metabólicos como la hiperglucemia (Leproult *et al.*, 2014), la resistencia a la insulina (Rao *et al.*, 2015) o la dislipidemia (Chua *et al.*, 2013).

Tanto diversas moléculas derivadas de los alimentos como los nutrientes liposolubles de la dieta pueden activar los factores de transcripción de la superfamilia de los receptores nucleares, tales como PPARs, REV-ERBs y RORs, entre otros muchos, siendo considerados además como puntos de unión clave entre el sistema circadiano y el metabolismo (Duez y Staels, 2009; Solt *et al.*, 2011; Chen y Yang, 2014; Vieira *et al.*, 2015; Ribas-Latre y Eckel-Mahan, 2016; Albrecht y Ripperger, 2018). En el carpín, observamos que *pparα* no presenta un ritmo diario de expresión en el hipotálamo ni en presencia de fotoperiodo con diferentes horarios de alimentación (ZT2, ZT6, ZT18 o aleatorio; **Tabla 3**) ni cuando se mantuvo a los animales bajo oscuridad constante y alimentación fija a ZT2. Sin embargo, en los tejidos periféricos analizados (bulbo intestinal, intestino anterior e hígado) presenta una expresión rítmica diaria, lo que nos hace pensar que las funciones de las NAEs (también rítmicas en estos tejidos, como se discutió previamente) podrían desempeñarse de forma circadiana. Además, cuando la alimentación se realiza de forma fija (ya sea a ZT2, a ZT6 o a ZT18), podemos observar que las acrofases de este receptor nuclear se disponen en todos los casos unas horas previas al momento de la alimentación, a diferencia de lo observado en el hígado tanto del pez cebra (Paredes *et al.*, 2015) como de la dorada (Paredes *et al.*, 2014), en donde las acrofases se

disponían en la segunda mitad de la escotofase independientemente del horario de alimentación. Por su parte, en los roedores nocturnos rata y ratón, este receptor nuclear presenta sus variaciones de expresión con un incremento a lo largo del día y su máximo al inicio de la noche (Yang *et al.*, 2006; Chen *et al.*, 2010; Wang *et al.*, 2014). Nuestros datos corroboran lo que se observa en los mamíferos en donde la expresión de *pparα* aumenta en los momentos de ayuno (Liu *et al.*, 2014), durante los cuales los animales obtienen la energía necesaria incrementando la β-oxidación de los ácidos grasos en el hígado con la consecuente formación de cuerpos cetónicos (Ribas-Latre y Eckel-Mahan, 2016). Y es precisamente PPARα el que estimula esta β-oxidación de los ácidos grasos y el catabolismo de los lípidos en los mamíferos (Charoensuksai y Xu, 2010; Chen y Yang, 2014; Liu *et al.*, 2014), por lo que nuestros resultados parecen sugerir que este receptor nuclear podría estar desempeñando las mismas funciones en los peces teleósteos. En cambio, cuando la alimentación de los carpines se realizó de forma aleatoria (con la última toma a ZT2), la acrofase de *pparα* en el hígado de estos peces tiene lugar al final del periodo nocturno, estando adelantada 2 horas respecto a los animales alimentados de forma fija a ZT2. Al igual que ocurría con los genes reloj *per*, parece ser que una sola toma de alimento empieza a resetear el reloj hepático aunque no completamente, lo que vuelve a sugiere que la comida es un *input* muy fuerte para el hígado del carpín.

En los mamíferos, se ha demostrado que PPARα regula la transcripción de los genes *bmal1* y *rev-erbα*, ambos muy ligados tanto al sistema circadiano como al metabolismo. Por un lado, *bmal1* es uno de los genes reloj del bucle principal que controla los osciladores endógenos, aunque también es un factor lipogénico que presenta ritmos diarios de expresión mostrando su acrofase después de la comida (Shimba *et al.*, 2005, 2011; Zhang D *et al.*, 2014). Estableciendo una similitud con nuestros resultados, se demuestra que este gen se comporta de forma similar a los mamíferos, con un máximo de expresión en el hígado del carpín horas después del momento de la alimentación bajo todos los horarios de alimentación (ZT2, ZT6, ZT18 y aleatorio; **Tabla 3**), por lo que podría estar íntimamente relacionado con el metabolismo de los lípidos desarrollando funciones lipogénicas en los peces teleósteos. Por su parte, cabe destacar que *rev-erbα* es el único receptor nuclear que presenta ritmos diarios de expresión en el hipotálamo del carpín cuando el fotoperiodo está presente, independientemente del horario de alimentación, perdiéndose su ritmo en oscuridad constante. Esto parece indicar que REV-ERBα puede tener un papel más relevante en la regulación del oscilador de lo que se pensaba hasta ahora, actuando como un indicador del correcto funcionamiento del reloj endógeno. Además, este gen interviene en el metabolismo glucídico durante las fases de alimentación-ayuno en los mamíferos, de tal forma que

disminuye la expresión de los genes que codifican para enzimas clave del metabolismo de la glucosa, como es el caso de la PEPCK y la G6Pasa (Yin *et al.*, 2007; Berthier *et al.*, 2018). Estas enzimas son las encargadas de mantener el aporte necesario de glucosa durante los momentos de ayuno de los animales. Observando nuestros resultados, el receptor nuclear *rev-erbα* tanto en el hipotálamo como en el hígado del carpín presenta su máximo de expresión en mitad del periodo nocturno cuando la alimentación fue durante la fase diurna (fija a ZT2 o ZT6 o aleatoria con la última toma a ZT2), momento en el cual los peces se encuentran en ayuno con los niveles de glucosa circulante en sus valores más bajos, lo que concuerda perfectamente con lo descrito anteriormente en los mamíferos. Sin embargo, cuando la alimentación tuvo lugar por la noche (ZT18), la acrofase del ritmo de *rev-erbα* en el hipotálamo solamente se adelantó 3 horas respecto a las obtenidas con una alimentación diurna, mientras que en el hígado se produjo una inversión total pasando a tener su acrofase en mitad del día (ZT7,4). Todos estos resultados refuerzan nuestra hipótesis de que el hipotálamo es un clásico LEO y el hígado un FEO.

Por último, RORα también regula una gran cantidad de procesos metabólicos tanto relacionados con los lípidos como con la glucosa (Solt *et al.*, 2011; Kojetin y Burris, 2014). En nuestros resultados, la expresión de *rorα* sólo fue rítmica en el hipotálamo y bajo condiciones de fotoperiodo 12L:12D con alimentación fija a ZT2. En este caso, la acrofase de este receptor nuclear tuvo lugar unas horas después de la alimentación, lo que podría estar ayudando al pico máximo de glucosa circulante que tiene lugar también de forma inmediatamente postprandial (1 hora). Estos resultados concuerdan con lo descrito previamente en los mamíferos, ya que se ha visto que *rorα* es necesario para mantener unos niveles normales de glucosa plasmática (Kang *et al.*, 2007).

Por otra parte, no es un hecho aislado que los niveles máximos circulantes de glucosa ocurran unas horas después de la alimentación tanto con alimentación fija a ZT2 o aleatoria (con la última toma a ZT2) en presencia o no de un fotoperiodo 12L:12D, y que solamente presenten ritmos diarios significativos en presencia de uno de los dos *zeitgeber*s (ciclo luz-oscuridad o alimentación). En realidad, se observó que la alimentación es imprescindible para el mantenimiento/aparición del ritmo, ya que los niveles plasmáticos en peces mantenidos durante 25 h en ayuno fueron significativamente más bajos que los obtenidos en los animales alimentados, exhibiéndose ritmos circadianos en todos los grupos experimentales cuando no se tuvo en cuenta el ayuno para el análisis estadístico circadiano. Estos datos sugieren que los ritmos diarios de glucosa se encuentran controlados principalmente por la ingestión de alimentos en los peces, al igual que en los mamíferos (Kasanen *et al.*, 2018). En este sentido,

también esperábamos obtener resultados similares en la expresión de los receptores nucleares estudiados. Sin embargo, los receptores nucleares no presentaron diferencias entre los peces en ayunas frente a los alimentados, por lo que parece ser que no se encuentran regulados por los *zeitgeber*s de forma directa. Todos los datos expuestos en la presente Tesis Doctoral sugieren que los tres receptores nucleares estudiados actúan en la misma línea pero con ligeras diferencias. Así, REV-ERBα parece funcionar como un auténtico *output* o salida del sistema circadiano ya que presenta ritmos diarios solamente cuando también los presentan los genes reloj tanto en el hipotálamo como en el hígado del carpín, de tal forma que su expresión se encuentra controlada por el reloj endógeno sin enmascaramiento por el ciclo de alimentación-ayuno. Esto, junto con sus importantes funciones sobre el metabolismo, parece indicar que este receptor nuclear juega un papel muy importante en la regulación de la homeostasis temporal. Por su parte, PPARα parece estar también controlado por la ritmicidad del oscilador endógeno, aunque no tan estrictamente como en el caso de REV-ERBα; mientras que RORα parece que no tiene un papel tan importante en cuanto al control de la ritmicidad en los peces como lo tiene en los mamíferos. En la presente Tesis Doctoral hemos demostrado que los ritmos de los receptores nucleares que pueden señalizar NAEs u otras moléculas, las cuales indican el estatus energético de los peces, son dependientes del reloj endógeno.

De forma general, los resultados obtenidos en la presente Tesis Doctoral evidencian que los cambios en los principales *zeitgeber*s (ciclos luz-oscuridad y alimentación-ayuno) alteran los circuitos de los relojes endógenos en función del tejido analizado en el carpín, ya sean osciladores centrales (hipotálamo) o periféricos (tejido interrenal e hígado). De esta manera, el hipotálamo y el tejido interrenal del carpín se encuentran principalmente sincronizados por el ciclo luz-oscuridad, mientras que el ciclo alimentación-ayuno es el sincronizador más potente a nivel del hígado. Además, un desfase en el horario de la alimentación desajusta la sincronización de los relojes endógenos en el hipotálamo, el tejido interrenal y el hígado, por lo que la pérdida de la homeostasis temporal puede afectar negativamente a la fisiología del carpín. Adicionalmente, los receptores nucleares se están evidenciando como moléculas muy importantes para el mantenimiento de la homeostasis temporal en los peces teleósteos, funcionando como auténticos *outputs* o moléculas clave de salida del sistema circadiano controladas por los osciladores endógenos (**Figura 21**).

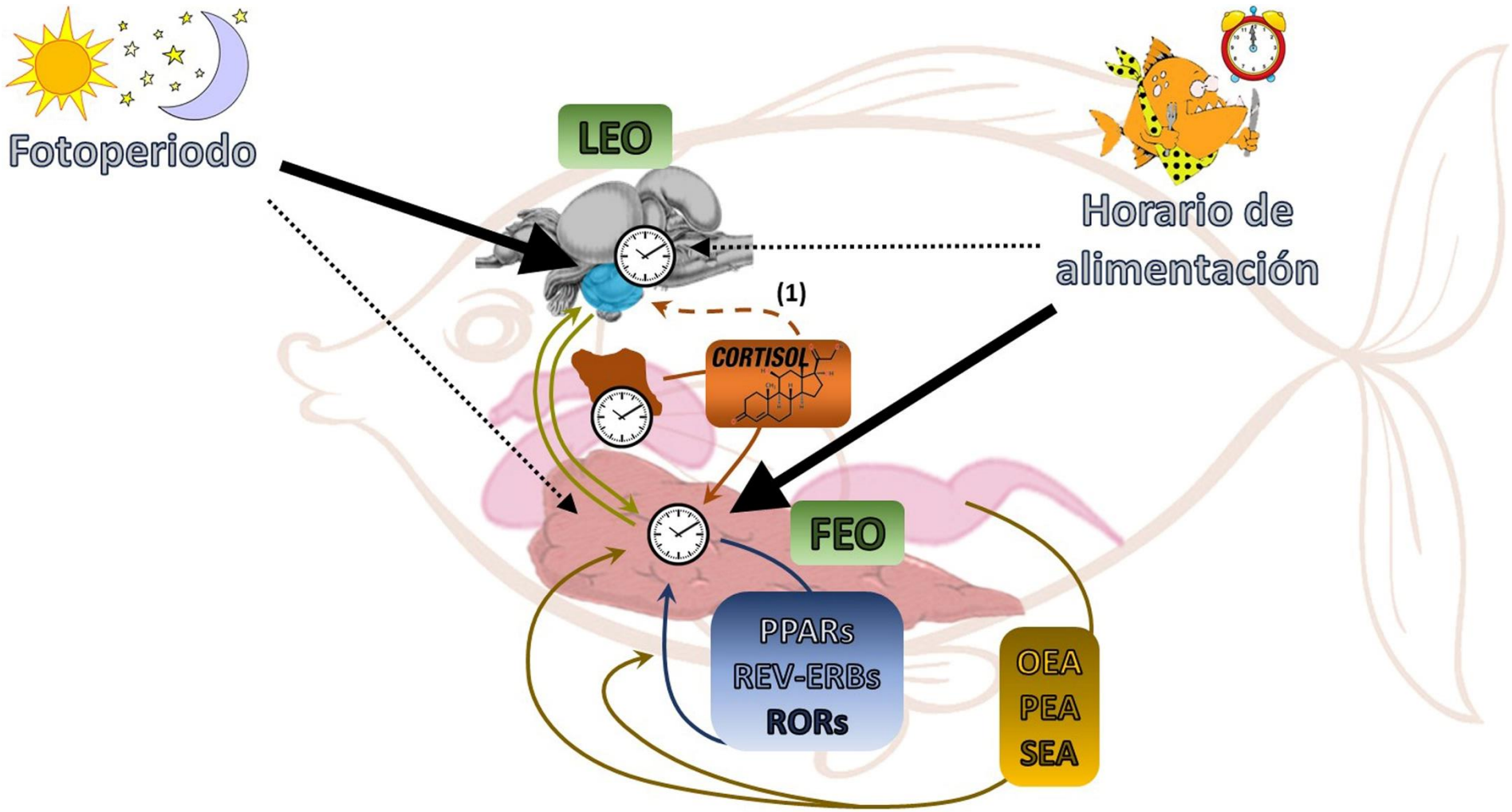


Figura 21. Representación esquemática de la sincronización del sistema circadiano por los diferentes *zeitgeber*s e integración de las señales rítmicas. FEO, oscilador sincronizado por la alimentación; LEO, oscilador sincronizado por la luz; OEA, oleiletanolamida; PEA, palmitoiletanolamida; PPAR, *peroxisome proliferator-activated receptor*; SEA, estearoiletanolamida; REV-ERB, *nuclear receptor subfamily 1 member D* o *nr1d*; ROR, *retinoic acid orphan receptor*. (1) Sánchez-Bretaño *et al.* (2016).


CONCLUSIONES /
CONCLUSIONS

CONCLUSIONES

A partir de los resultados obtenidos y discutidos en la presente Tesis Doctoral, proponemos las siguientes conclusiones:

1. La presencia en el carpín de todos los componentes del sistema de las NAEs: OEA, PEA y SEA, sus precursores, las enzimas de síntesis (NAPE-PLD) y de degradación (FAAH) y su receptor (PPARα), sugiere que estos derivados lipídicos actúan como moléculas bioactivas en los peces, pudiendo intervenir en la regulación de diferentes funciones fisiológicas, y apoyando la existencia de un sistema endógeno funcional de las NAEs a lo largo de la evolución de los vertebrados.
2. La alimentación es un importante regulador de las NAEs en los peces, como lo demuestran las importantes variaciones en los niveles gastrointestinales de OEA, PEA y SEA, en respuesta a los ciclos diarios de alimentación y ayuno. El rápido aumento postprandial observado en tejidos gastrointestinales, pero no en tejidos cerebrales, sugiere que estas NAEs podrían estar actuando como señales periféricas de saciedad a corto plazo.
3. La PEA presenta un efecto anorético y además reduce el peso corporal, efectos que podrían estar mediados por un aumento de la leptina hepática y una disminución del NPY hipotalámico. La acusada reducción de la actividad anticipatoria a la alimentación apoya un efecto de la PEA no solo en la etapa consumatoria del comportamiento alimentario, sino también en etapas previas de alerta y localización de la comida.
4. Se han obtenido evidencias sobre un papel de las NAEs en la regulación del metabolismo hepático en los peces. Cabe destacar un efecto lipogénico inducido por OEA y PEA, acompañado de la consiguiente reducción del potencial lipolítico en el caso de la OEA, o sin modificaciones en el caso de la PEA. En relación al metabolismo glucídico, nuestros resultados apoyan una importante reducción en el potencial gluconeogénico por ambas

NAEs, así como un incremento del potencial glucogenolítico. Dichos efectos de la OEA y la PEA en el metabolismo hepático podrían estar mediados por el aumento de la expresión de *bmal1a* que inducen ambas NAEs en el hígado.

5. El hecho de que los ritmos de genes reloj en el hipotálamo se pierdan cuando se elimina el *zeitgeber* luz-oscuridad, y que los cambios en el horario de la alimentación apenas les afecten, nos permite proponer que en el carpín el hipotálamo se comporta como un oscilador sincronizado por la luz (LEO). Por su parte, el hecho de que en el hígado todos los genes reloj estudiados se desfasen 12 h cuando se desfasa 12 h el horario de la comida, así como que dichos ritmos se mantengan en condiciones de curso libre (oscuridad continua) siempre que haya un horario fijo de alimentación, nos permite proponer que el hígado se comporta como un oscilador sincronizado por la comida (FEO) en esta especie.
6. Existen ritmos diarios de las NAEs (OEA, PEA y SEA) y de la expresión de su receptor principal (PPARα) en el hígado y el intestino del carpín asociados al momento de la ingesta. Esto hace pensar que estos derivados lipídicos puedan señalizar la hora de la comida pudiendo actuar como entradas o *inputs* de los FEOs. En este sentido hemos demostrado que tanto la OEA como la PEA modulan los genes reloj en el hígado, aunque si son capaces de sincronizarlo es aún una incógnita. El hecho de que no se hayan encontrado ritmos de las NAEs asociados a hora de la comida en el hipotálamo apoya esta idea, ya que este tejido es un LEO en el carpín.
7. La glándula interrenal parece ser más sensible al ciclo luz-oscuridad, sugiriendo que se comporta más como un LEO en el carpín. El desajuste entre los *zeitgeber*s ciclos luz-oscuridad y alimentación-ayuno afecta al oscilador del tejido interrenal en mayor medida que a los osciladores hipotalámico y hepático, ya que se pierden los desfases esperables entre los genes reloj de la mitad positiva y los de la negativa del bucle principal, así como el ritmo diario de cortisol. Todo ello apoya que una alteración de la homeostasis temporal es un agente estresante para los peces.
8. De los receptores nucleares estudiados (REV-ERB, PPAR y ROR), la expresión de *rev-erbα* es la que parece tener ritmos diarios más robustos. La expresión rítmica de este receptor nuclear se encuentra ligada a la existencia de ritmos en los genes reloj del bucle principal, estando por tanto controlada por el ciclo luz-oscuridad en el hipotálamo y por la alimentación en el hígado. Estos resultados demuestran que en el carpín *rev-erbα* es un gen controlado por el reloj (CCG) y que, tal como se ha propuesto recientemente en mamíferos, su papel para el funcionamiento del oscilador es más importante de lo que hasta hace poco se creía.

9. La existencia de ritmos diarios en la expresión de *rev-erbβ*, *rorα* y *pparα* en el hígado, aunque no en el hipotálamo, sugiere que la ritmicidad de estos receptores nucleares es probablemente más importante para el metabolismo hepático que para otras funciones. Sin embargo, y en contra de lo esperado, no hay cambios postprandiales (a corto plazo) en la expresión de ninguno de los receptores nucleares estudiados, aunque no podemos descartar cambios en respuesta a ayunos más prolongados.

CONCLUSIONS

Based on the results obtained and discussed in the present Doctoral Thesis, we propose the following conclusions:

1. Presence of all components of the NAEs' system: OEA, PEA, and SEA, their precursors, the synthesis (NAPE-PLD) and degradation (FAAH) enzymes, and their main receptor (PPARα), suggests that these lipid derivates act as bioactive molecules in fish, being able to participate in the regulation of different physiological functions, and supporting the existence of a functional endogenous system of NAEs throughout the evolution of vertebrates.
2. Food intake is a key regulator of NAEs in fish, as demonstrated by the significant variations in gastrointestinal levels of OEA, PEA, and SEA, in response to daily feeding and fasting cycles. The quick postprandial increase observed in gastrointestinal tissues, but not in brain ones, suggests that these NAEs may be acting as short-term peripheral satiety signals.
3. PEA has an anorectic effect and also reduces body weight, effects that may be mediated by the increase in hepatic leptin and the decrease in hypothalamic NPY. The sharp reduction of the food anticipatory activity supports an effect of PEA not only on the feeding-behavior consummatory stage, but also in the awareness and location of food.
4. Evidences have been obtained for the role of NAEs in the regulation of hepatic metabolism in fish. A lipogenic effect induced by OEA and PEA should be noted, linked to a reduction of the lipolytic potential in the case of OEA, or no modification in the case of PEA. Related to glucose metabolism, our results endorse a significant reduction in the gluconeogenic potential by both NAEs, as well as an increased potential of glycogenolysis. Those OEA and PEA effects on hepatic metabolism may be mediated by the NAEs-induced increase in the *bmal1a* expression in the liver.

5. The fact that the clock genes rhythms in the hypothalamus are lost when the light-dark cycle is removed, and that changes in the scheduled feeding barely affect them, allow us to propose that the hypothalamus in goldfish behaves as a light-entrainable oscillator (LEO). As for the liver, the fact that the 12 h shift in the scheduled feeding induced a 12 h shift in all the studied clock genes, as well as their rhythms are maintained under free-running conditions (constant darkness) with a scheduled feeding, allow us to propose that the liver behaves as a food-entrainable oscillator (FEO) in this species.
6. There are daily rhythms of NAEs (OEA, PEA, and SEA) and of the expression of their main receptor (PPARα) in the liver and intestine of the goldfish linked to the feeding time. This suggests that these lipid derivatives can signal the mealtime and can act as inputs of the FEOs. In this sense, we have demonstrated that both OEA and PEA modulate the clock genes in the liver, although if they are capable to synchronize them is still unknown. The fact that no NAEs rhythms associated with mealtime have been found in the hypothalamus reinforces this idea, as this tissue is a LEO in the goldfish.
7. The interrenal gland seems to be more sensitive to the light-dark cycle, suggesting that it behaves more like a LEO in the goldfish. The mismatch between the *zeitgeber*s light-dark and feeding-fasting cycles affects the interrenal tissue oscillator in a stronger way than the hypothalamic and hepatic oscillators, as the genes of the positive arm and of the negative one of the main loop are not in phase, as well as the loss of the daily rhythm of cortisol. All these findings support that an alteration of the temporal homeostasis is a stressor for fish.
8. Among the studied nuclear receptors (REV-ERB, PPAR, and ROR), the expression of *rev-erbα* seems to display the most robust daily rhythms. The rhythmic expression of this nuclear receptor is linked to the existence of rhythms of the main loop clock genes, being therefore controlled by the light-dark cycle in the hypothalamus and by feeding in the liver. These results show that in goldfish *rev-erbα* is a clock-controlled gene (CCG) and that, as recently proposed in mammals, its role for oscillator functioning is more important than it was previously believed.
9. The existence of daily expression rhythms of *rev-erbβ*, *rorα*, and *pparα* in the liver, but not in the hypothalamus, suggests that the rhythmicity of these nuclear receptors is probably more relevant for the hepatic metabolism than for other functions. However, contrary to expectations, there are no (short-term) postprandial changes in the gene expression of any studied nuclear receptors, although we cannot rule out changes in response to longer fasting periods.

BIBLIOGRAFÍA

A

Abbott M y Volkoff H (2011) Thyrotropin Releasing Hormone (TRH) in goldfish (*Carassius auratus*): Role in the regulation of feeding and locomotor behaviors and interactions with the orexin system and cocaine- and amphetamine regulated transcript (CART). *Horm Behav* **59**:236–245.

Adamovich Y, Aviram R y Asher G (2015) The emerging roles of lipids in circadian control. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids* **1851**:1017–1025.

Aguilar AJ, Conde-Sieira M, López-Patiño MA, Míguez JM y Soengas JL (2011) *In vitro* leptin treatment of rainbow trout hypothalamus and hindbrain affects glucosensing and gene expression of neuropeptides involved in food intake regulation. *Peptides* **32**:232–240.

Albrecht U (2012) Timing to perfection: the biology of central and peripheral circadian clocks. *Neuron* **74**:246–260.

Albrecht U y Ripperger JA (2018) Circadian clocks and sleep: Impact of rhythmic metabolism and waste clearance on the brain. *Trends Neurosci* **41**:677–688.

Allaman-Pillet N, Roduit R, Oberson A, Abdelli S, Ruiz J, Beckmann JS, Schorderet DF y Bonny C (2004) Circadian regulation of islet genes involved in insulin production and secretion. *Mol Cell Endocrinol* **226**:59–66.

Apromar (2018) *Informe La Acuicultura en España*, Ediciones APROMAR, España.

Aranda A, Madrid JA y Sánchez-Vázquez FJ (2001) Influence of light on feeding anticipatory activity in goldfish. *J Biol Rhythms* **16**:50–57.

Artmann A, Petersen G, Hellgren LI, Boberg J, Skonberg C, Nellemann C, Hansen SH y Hansen HS (2008) Influence of dietary fatty acids on endocannabinoid and N-acylethanolamine levels in rat brain, liver and small intestine. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids* **1781**:200–212.

Aschoff J (1981) A survey on biological rhythms. En: *Biological Rhythms* (Aschoff J, ed), Springer, Boston, MA, USA. pp 3–10.

Asher G y Schibler U (2011) Crosstalk between components of circadian and metabolic cycles in mammals. *Cell Metab* **13**:125–137.

Astarita G, Rourke BC, Andersen JB, Fu J, Kim JH, Bennett AF, Hicks JW y Piomelli D (2006) Postprandial increase of oleoylethanolamide mobilization in small intestine of the Burmese python (*Python molurus*). *Am J Physiol-Regul Integr Comp Physiol* **290**:R1407–R1412.

Azari EK, Ramachandran D, Weibel S, Arnold M, Romano A, Gaetani S, Langhans W y Mansouri A (2014) Vagal afferents are not necessary for the satiety effect of the gut lipid messenger oleoylethanolamide. *Am J Physiol-Regul Integr Comp Physiol* **307**:R167–R178.

Azpeleta C, Martínez-Álvarez RM, Delgado MJ, Isorna E y de Pedro N (2010) Melatonin reduces locomotor activity and circulating cortisol in goldfish. *Horm Behav* **57**:323–329.

B

Balasubramanian MN, Panserat S, Dupont-Nivet M, Quillet E, Montfort J, Le Cam A, Medale F, Kaushik SJ y Geurden I (2016) Molecular pathways associated with the nutritional programming of plant-based diet acceptance in rainbow trout following an early feeding exposure. *BMC Genomics* **17**:449.

Baldwin WS, Boswell WT, Ginjupalli G y Litoff EJ (2017) Annotation of the nuclear receptors in an estuarine fish species, *Fundulus heteroclitus*. *Nucl Recept Res* **4**:101285.

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schütz G y Schibler U (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* **289**:2344–2347.

Bass J y Takahashi JS (2010) Circadian integration of metabolism and energetics. *Science* **330**:1349–1354.

Beale A, Guibal C, Tamai TK, Klotz L, Cowen S, Peyric E, Reynoso VH, Yamamoto Y y Whitmore D (2013) Circadian rhythms in Mexican blind cavefish *Astyanax mexicanus* in the lab and in the field. *Nat Commun* **4**:2769.

Bechtold DA y Loudon ASI (2013) Hypothalamic clocks and rhythms in feeding behaviour. *Trends Neurosci* **36**:74–82.

Ben-Moshe Z, Alon S, Mracek P, Faigenbloom L, Tovin A, Vatine GD, Eisenberg E, Foulkes NS y Gothilf Y (2014) The light-induced transcriptome of the zebrafish pineal gland reveals complex regulation of the circadian clockwork by light. *Nucleic Acids Res* **42**:3750–3767.

Berger J y Moller DE (2002) The mechanisms of action of PPARs. *Annu Rev Med* **53**:409–435.

Bernier NJ y Peter RE (2001) Appetite-suppressing effects of urotensin I and corticotropin-releasing hormone in goldfish (*Carassius auratus*). *Neuroendocrinology* **73**:248–260.

Bernier NJ, Bedard N y Peter RE (2004) Effects of cortisol on food intake, growth, and forebrain neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. *Gen Comp Endocrinol* **135**:230–240.

Berthier A, Vinod M, Porez G, Steenackers A, Alexandre J, Yamakawa N, Gheeraert C, Ploton M, Maréchal X, Dubois-Chevalier J, Hovasse A, Schaeffer-Reiss C, Cianférani S, Rolando C, Bray F, Duez H, Eeckhoute J, Lefebvre T, Staels B y Lefebvre P (2018) Combinatorial

regulation of hepatic cytoplasmic signaling and nuclear transcriptional events by the OGT/REV-ERBα complex. *Proc Natl Acad Sci* **115**:E11033–E11042.

Bertucci JI, Blanco AM, Sundarrajan L, Rajeswari JJ, Velasco C y Unniappan S (2019) Nutrient regulation of endocrine factors influencing feeding and growth in fish. *Front Endocrinol* **10**:83.

Betancor MB, McStay E, Minghetti M, Migaud H, Tocher DR y Davie A (2014) Daily rhythms in expression of genes of hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). *PLoS ONE* **9**:e106739.

Betancor MB, Ortega A, de la Gándara F, Tocher DR y Mourente G (2017) Lipid metabolism-related gene expression pattern of Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae fed on live prey. *Fish Physiol Biochem* **43**:493–516.

Blanco AM, Gómez-Boronat M, Redondo I, Valenciano AI y Delgado MJ (2016) Periprandial changes and effects of short- and long-term fasting on ghrelin, GOAT, and ghrelin receptors in goldfish (*Carassius auratus*). *J Comp Physiol B* **186**:727–738.

Blanco AM, Sundarrajan L, Bertucci JI y Unniappan S (2018) Why goldfish? Merits and challenges in employing goldfish as a model organism in comparative endocrinology research. *Gen Comp Endocrinol* **257**:13–28.

Blomqvist AG, Söderberg C, Lundell I, Milner RJ y Larhammar D (1992) Strong evolutionary conservation of neuropeptide Y: sequences of chicken, goldfish, and *Torpedo marmorata* DNA clones. *Proc Natl Acad Sci U S A* **89**:2350–2354.

Borrelli F e Izzo AA (2009) Role of acylethanolamides in the gastrointestinal tract with special reference to food intake y energy balance. *Best Pract Res Clin Endocrinol Metab* **23**:33–49.

Borrelli F, Romano B, Petrosino S, Pagano E, Capasso R, Coppola D, Battista G, Orlando P, Di Marzo V y Izzo AA (2015) Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent. *Br J Pharmacol* **172**:142–158.

Bosler O, Girardet C, Franc J-L, Becquet D y François-Bellan A-M (2015) Structural plasticity of the circadian timing system. An overview from flies to mammals. *Front Neuroendocrinol* **38**:50–64.

Bottemanne P, Muccioli GG y Alhouayek M (2018) N-acylethanolamine hydrolyzing acid amidase inhibition: tools and potential therapeutic opportunities. *Drug Discov Today* **23**:1520–1529.

Bowen KJ, Kris-Etherton PM, Shearer GC, West SG, Reddivari L y Jones PJH (2017) Oleic acid-derived oleoylethanolamide: A nutritional science perspective. *Prog Lipid Res* **67**:1–15.

Bozek K, Relógio A, Kielbasa SM, Heine M, Dame C, Kramer A y Herzel H (2009) Regulation of clock-controlled genes in mammals. *PLOS ONE* **4**:e4882.

Brown JD, Karimian Azari E y Ayala JE (2017) Oleoylethanolamide: A fat ally in the fight against obesity. *Physiol Behav* **176**:50–58.

Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N y Schibler U (2002) Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr Biol* **12**:1574–1583.

Bruun S, Gouveia-Figueira S, Domellöf M, Husby S, Neergaard Jacobsen L, Michaelsen KF, Fowler CJ y Zachariassen G (2018) Satiety factors oleoylethanolamide, stearoylethanolamide, and palmitoylethanolamide in mother's milk are strongly associated with infant weight at four months of age—Data from the Odense child cohort. *Nutrients* **10**:1747.

Buhr ED y Takahashi JS (2013) Molecular components of the mammalian circadian clock. *Handb Exp Pharmacol* 3–27.

Buhr ED, Yoo S-H y Takahashi JS (2010) Temperature as a universal resetting cue for mammalian circadian oscillators. *Science* **330**:379–385.

C

Cadas H, Tomaso E di y Piomelli D (1997) Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain. *J Neurosci* **17**:1226–1242.

Cahill GM (2002) Clock mechanisms in zebrafish. *Cell Tissue Res* **309**:27–34.

Cahill GM y Besharse JC (1991) Resetting the circadian clock in cultured *Xenopus* eyecups: regulation of retinal melatonin rhythms by light and D2 dopamine receptors. *J Neurosci* **11**:2959–2971.

Cahill GM y Besharse JC (1993) Circadian clock functions localized in xenopus retinal photoreceptors. *Neuron* **10**:573–577.

Cambras Riu T (2006) Propiedades fundamentales de los ritmos circadianos. En: *Cronobiología básica y clínica* (Madrid Pérez JA y Rol de Lama Á, eds), Editec @Red, Madrid, España. pp 151–189.

Campolongo P, Roozendaal B, Trezza V, Cuomo V, Astarita G, Fu J, McGaugh JL y Piomelli D (2009) Fat-induced satiety factor oleoylethanolamide enhances memory consolidation. *Proc Natl Acad Sci U S A* **106**:8027–8031.

Canaple L, Rambaud J, Dkhissi-Benyahya O, Rayet B, Tan NS, Michalik L, Delaunay F, Wahli W y Laudet V (2006) Reciprocal regulation of brain and muscle Arnt-like protein 1 and peroxisome proliferator-activated receptor α defines a novel positive feedback loop in the rodent liver circadian clock. *Mol Endocrinol* **20**:1715–1727.

Cani PD, Montoya ML, Neyrinck AM, Delzenne NM y Lambert DM (2004) Potential modulation of plasma ghrelin and glucagon-like peptide-1 by anorexigenic cannabinoid compounds, SR141716A (rimonabant) and oleoylethanolamide. *Br J Nutr* **92**:757–761.

Canosa LF, Unniappan S y Peter RE (2005) Periprandial changes in growth hormone release in goldfish: role of somatostatin, ghrelin, and gastrin-releasing peptide. *Am J Physiol-Regul Integr Comp Physiol* **289**:R125–R133.

Capasso R y Izzo AA (2008) Gastrointestinal regulation of food intake: General aspects and focus on anandamide and oleoylethanolamide. *J Neuroendocrinol* **20**:39–46.

Carmona-Antoñanzas G, Tocher DR, Martinez-Rubio L y Leaver MJ (2014) Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. *Gene* **534**:1–9.

Caruso MA y Sheridan MA (2011) New insights into the signaling system and function of insulin in fish. *Gen Comp Endocrinol* **173**:227–247.

Castañeda TR, Tong J, Datta R, Culler M y Tschöp MH (2010) Ghrelin in the regulation of body weight and metabolism. *Front Neuroendocrinol* **31**:44–60.

Cavallari N, Frigato E, Vallone D, Fröhlich N, Lopez-Olmeda JF, Foà A, Berti R, Sánchez-Vázquez FJ, Bertolucci C y Foulkes NS (2011) A blind circadian clock in cavefish reveals that opsins mediate peripheral clock photoreception. *PLOS Biol* **9**:e1001142.

Ceinos RM, Frigato E, Pagano C, Fröhlich N, Negrini P, Cavallari N, Vallone D, Fuselli S, Bertolucci C y Foulkes NS (2018) Mutations in blind cavefish target the light-regulated circadian clock gene, *period 2*. *Sci Rep* **8**.

Cerdá-Reverter JM y Larhammar D (2000) Neuropeptide Y family of peptides: structure, anatomical expression, function, and molecular evolution. *Biochem Cell Biol Biochim Biol Cell* **78**:371–392.

Cerdá-Reverter JM y Peter RE (2003) Endogenous melanocortin antagonist in fish: Structure, brain mapping and regulation by fasting of the goldfish agouti-related protein gene. *Endocrinology* **144**:4552–4561.

Cerdá-Reverter JM, Schiöth HB y Peter RE (2003) The central melanocortin system regulates food intake in goldfish. *Regul Pept* **115**:101–113.

Chakravarthy MV, Lodhi IJ, Yin L, Malapaka RRV, Xu HE, Turk J y Semenkovich CF (2009) Identification of a physiologically relevant endogenous ligand for PPARα in liver. *Cell* **138**:476–488.

Challet E (2015) Keeping circadian time with hormones. *Diabetes Obes Metab* **17**:76–83.

Charoensuksai P y Xu W (2010) PPARs in rhythmic metabolic regulation and implications in health and disease. *PPAR Res* **2010**.

Chen L y Yang G (2014) PPARs integrate the mammalian clock and energy metabolism. *PPAR Res* **2014**.

Chen P, Kakan X y Zhang J (2010) Altered circadian rhythm of the clock genes in fibrotic livers induced by carbon tetrachloride. *FEBS Lett* **584**:1597–1601.

Chisada S, Kurokawa T, Murashita K, Rønnestad I, Taniguchi Y, Toyoda A, Sakaki Y, Takeda S y Yoshiura Y (2014) Leptin receptor-deficient (knockout) medaka, *Oryzias latipes*, show

chronical up-regulated levels of orexigenic neuropeptides, elevated food intake and stage specific effects on growth and fat allocation. *Gen Comp Endocrinol* **195**:9–20.

Cho H, Zhao X, Hatori M, Yu RT, Barish GD, Lam MT, Chong L-W, DiTacchio L, Atkins AR, Glass CK, Liddle C, Auwerx J, Downes M, Panda S y Evans RM (2012) Regulation of circadian behavior and metabolism by *rev-erbα* and *rev-erbβ*. *Nature* **485**:123–127.

Cho HK, Kong HJ, Kim HY y Cheong J (2012) Characterization of *Paralichthys olivaceus* peroxisome proliferator-activated receptor-α gene as a master regulator of flounder lipid metabolism. *Gen Comp Endocrinol* **175**:39–47.

Chua EC-P, Shui G, Lee IT-G, Lau P, Tan L-C, Yeo S-C, Lam BD, Bulchand S, Summers SA, Puvanendran K, Rozen SG, Wenk MR y Gooley JJ (2013) Extensive diversity in circadian regulation of plasma lipids and evidence for different circadian metabolic phenotypes in humans. *Proc Natl Acad Sci* **110**:14468–14473.

Citraro R, Russo E, Scicchitano F, van Rijn CM, Cosco D, Avagliano C, Russo R, D'Agostino G, Petrosino S, Guida F, Gatta L, van Luijtelaar G, Maione S, Di Marzo V, Calignano A y De Sarro G (2013) Antiepileptic action of N-palmitoylethanolamine through CB1 and PPAR-α receptor activation in a genetic model of absence epilepsy. *Neuropharmacology* **69**:115–126.

Comesaña S, Velasco C, Ceinos RM, López-Patiño MA, Míguez JM, Morais S y Soengas JL (2018) Evidence for the presence in rainbow trout brain of amino acid-sensing systems involved in the control of food intake. *Am J Physiol-Regul Integr Comp Physiol* **314**:R201–R215.

Conde-Sieira M y Soengas JL (2017) Nutrient sensing systems in fish: impact on food intake regulation and energy homeostasis. *Front Neurosci* **10**:21.

Costa LS, Serrano I, Sánchez-Vázquez FJ y López-Olmeda JF (2016) Circadian rhythms of clock gene expression in Nile tilapia (*Oreochromis niloticus*) central and peripheral tissues: influence of different lighting and feeding conditions. *J Comp Physiol B* 1–11.

Coulon D, Faure L, Salmon M, Wattelet V y Bessoule J-J (2012) Occurrence, biosynthesis and functions of N-acylphosphatidylethanolamines (NAPE): Not just precursors of N-acylethanolamines (NAE). *Biochimie* **94**:75–85.

Cowan M, Azpeleta C y López-Olmeda JF (2017) Rhythms in the endocrine system of fish: a review. *J Comp Physiol B* **187**:1057–1089.

Cummings DE (2006) Ghrelin and the short- and long-term regulation of appetite and body weight. *Physiol Behav* **89**:71–84.

Cummings DE y Overduin J (2007) Gastrointestinal regulation of food intake. *J Clin Invest* **117**:13–23.

Cunha I, Galante-Oliveira S, Rocha E, Planas M, Urbatzka R y Castro LFC (2013) Dynamics of PPARs, fatty acid metabolism genes and lipid classes in eggs and early larvae of a teleost. *Comp Biochem Physiol B Biochem Mol Biol* **164**:247–258.

D

D'Agostino G, La Rana G, Russo R, Sasso O, Iacono A, Esposito E, Mattace Raso G, Cuzzocrea S, LoVerme J, Piomelli D, Meli R y Calignano A (2009) Central administration of palmitoylethanolamide reduces hyperalgesia in mice via inhibition of NF-κB nuclear signalling in dorsal root ganglia. *Eur J Pharmacol* **613**:54–59.

Dalle Carbonare M, Del Giudice E, Stecca A, Colavito D, Fabris M, D'Arrigo A, Bernardini D, Dam M y Leon A (2008) A saturated N-acylethanolamine other than N-palmitoyl ethanolamine with anti-inflammatory properties: a neglected story…. *J Neuroendocrinol* **20**:26–34.

Dalmolin C, Almeida DV, Figueiredo MA y Marins LF (2015) Food intake and appetite control in a GH-transgenic zebrafish. *Fish Physiol Biochem* **41**:1131–1141.

Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F y Schibler U (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* **14**:2950–2961.

de Pedro N, Delgado MJ y Alonso-Bedate M (1995) Central administration of β-endorphin increases food intake in goldfish: pretreatment with the opioid antagonist naloxone. *Regul Pept* **55**:189–195.

de Pedro N, Céspedes MV, Delgado MJ y Alonso-Bedate M (1996) Mu-opioid receptor is involved in β-endorphin-induced feeding in goldfish. *Peptides* **17**:421–424.

de Pedro N, Delgado MJ, Pinillos ML y Alonso-Bedate M (1998a) α1-Adrenergic and dopaminergic receptors are involved in the anoretic effect of corticotropin-releasing factor in goldfish. *Life Sci* **62**:1801–1808.

de Pedro N, Pinillos ML, Valenciano AI, Alonso-Bedate M y Delgado MJ (1998b) Inhibitory effect of serotonin on feeding behavior in goldfish: Involvement of CRF. *Peptides* **19**:505–511.

de Pedro N, López-Patiño MA, Guijarro AI, Pinillos ML, Delgado MJ y Alonso-Bedate M (2000) NPY receptors and opioidergic system are involved in NPY-induced feeding in goldfish. *Peptides* **21**:1495–1502.

de Pedro N, Martínez-Álvarez R y Delgado MJ (2006) Acute and chronic leptin reduces food intake and body weight in goldfish (*Carassius auratus*). *J Endocrinol* **188**:513–520.

De Vriese C y Delporte C (2008) Ghrelin: A new peptide regulating growth hormone release and food intake. *Int J Biochem Cell Biol* **40**:1420–1424.

Decara JM, Romero-Cuevas M, Rivera P, Macias-González M, Vida M, Pavón FJ, Serrano A, Cano C, Fresno N, Pérez-Fernández R, Rodríguez de Fonseca F y Suárez J (2012) Elaidyl-sulfamide, an oleoylethanolamide-modelled PPARα agonist, reduces body weight gain and plasma cholesterol in rats. *Dis Model Mech* **5**:660–670.

Delaunay F, Thisse C, Marchand O, Laudet V y Thisse B (2000) An inherited functional circadian clock in zebrafish embryos. *Science* **289**:297–300.

Delerive P, Chin WW y Suen CS (2002) Identification of reverbα as a novel RORα target gene. *J Biol Chem* **277**:35013–35018.

Delgado MJ, Alonso-Gómez AL, Gancedo B, de Pedro N, Valenciano AI y Alonso-Bedate M (1993) Serotonin N-acetyltransferase (NAT) activity and melatonin levels in the frog retina are not correlated during the seasonal cycle. *Gen Comp Endocrinol* **92**:143–150.

Delgado MJ, Cerdá-Reverter JM y Soengas JL (2017) Hypothalamic integration of metabolic, endocrine and circadian signals in fish: Involvement in the control of food intake. *Front Neurosci* **11**:354.

Den Broeder MJ, Kopylova VA, Kamminga LM y Legler J (2015) Zebrafish as a model to study the role of peroxisome proliferating-activated receptors in adipogenesis and obesity. *PPAR Res* **2015**:11.

Dibner C y Schibler U (2015) Circadian timing of metabolism in animal models y humans. *J Intern Med* **277**:513–527.

Dibner C, Schibler U y Albrecht U (2010) The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol* **72**:517–549.

Diep TA, Madsen AN, Holst B, Kristiansen MM, Wellner N, Hansen SH y Hansen HS (2011) Dietary fat decreases intestinal levels of the anorectic lipids through a fat sensor. *FASEB J* **25**:765–774.

Du L, Yang X, Yang L, Wang X, Zhang A y Zhou H (2012) Molecular evidence for the involvement of *RORα* and *RORγ* in immune response in teleost. *Fish Shellfish Immunol* **33**:418–426.

Duez H y Staels B (2009) Rev-erb-α: an integrator of circadian rhythms and metabolism. *J Appl Physiol* **107**:1972–1980.

Duggleby RG (1981) A nonlinear regression program for small computers. *Anal Biochem* **110**:9–18.

E

Eckel-Mahan K y Sassone-Corsi P (2013) Metabolism and the circadian clock converge. *Physiol Rev* **93**:107–135.

Escobar S, Rocha A, Felip A, Carrillo M, Zanuy S, Kah O y Servili A (2016) Leptin receptor gene in the European sea bass (*Dicentrarchus labrax*): Cloning, phylogeny, tissue distribution and neuroanatomical organization. *Gen Comp Endocrinol* **229**:100–111.

Everett LJ y Lazar MA (2014) Nuclear receptor Rev-erbα: up, down, and all around. *Trends Endocrinol Metab* **25**:586–592.

F

Fahrenkrug J, Hannibal J y Georg B (2008) Diurnal rhythmicity of the canonical clock genes *Per1*, *Per2* y *Bmal1* in the rat adrenal gland is unaltered after hypophysectomy. *J Neuroendocrinol* **20**:323–329.

FAO (2018) *El estado mundial de la pesca y la acuicultura 2018. Cumplir los objetivos de desarrollo sostenible*. Roma, Licencia: CC BY-NC-SA 3.0 IGO.

Feliciano A, Vivas Y, Pedro N de, Delgado MJ, Velarde E e Isorna E (2011) Feeding time synchronizes clock gene rhythmic expression in brain and liver of goldfish (*Carassius auratus*). *J Biol Rhythms* **26**:24–33.

Ferrell JM y Chiang JYL (2015) Circadian rhythms in liver metabolism and disease. *Acta Pharm Sin B* **5**:113–122.

Flores MV, Hall C, Jury A, Crosier K y Crosier P (2007) The zebrafish retinoid-related orphan receptor (*ror*) gene family. *Gene Expr Patterns* **7**:535–543.

Fontaine C, Dubois G, Duguay Y, Helledie T, Vu-Dac N, Gervois P, Soncin F, Mandrup S, Fruchart J-C, Fruchart-Najib J y Staels B (2003) The orphan nuclear receptor Rev-Erbα is a peroxisome proliferator-activated receptor (PPAR) γ target gene and promotes PPARγ-induced adipocyte differentiation. *J Biol Chem* **278**:37672–37680.

Forner-Piquer I, Santangeli S, Maradonna F, Rabbito A, Piscitelli F, Habibi HR, Di Marzo V y Carnevali O (2018) Disruption of the gonadal endocannabinoid system in zebrafish exposed to diisononyl phthalate. *Environ Pollut* **241**:1–8.

Fredriksson R, Höglund PJ, Gloriam DEI, Lagerström MC y Schiöth HB (2003) Seven evolutionarily conserved human rhodopsin G protein-coupled receptors lacking close relatives. *FEBS Lett* **554**:381–388.

Fu J, Gaetani S, Oveisi F, LoVerme J, Serrano A, Rodríguez de Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G y Piomelli D (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. *Nature* **425**:90–93.

Fu J, Oveisi F, Gaetani S, Lin E y Piomelli D (2005) Oleoylethanolamide, an endogenous PPAR-α agonist, lowers body weight and hyperlipidemia in obese rats. *Neuropharmacology* **48**:1147–1153.

Fu J, Astarita G, Gaetani S, Kim J, Cravatt BF, Mackie K y Piomelli D (2007) Food intake regulates oleoylethanolamide formation and degradation in the proximal small intestine. *J Biol Chem* **282**:1518–1528.

Fu J, DiPatrizio NV, Guijarro A, Schwartz GJ, Li X, Gaetani S, Astarita G y Piomelli D (2011) Sympathetic activity controls fat-induced oleoylethanolamide signaling in small intestine. *J Neurosci* **31**:5730–5736.

G

Gaetani S, Fu J, Cassano T, Dipasquale P, Romano A, Righetti L, Cianci S, Laconca L, Giannini E, Scaccianoce S, Mairesse J, Cuomo V y Piomelli D (2010) The fat-induced satiety factor oleoylethanolamide suppresses feeding through central release of oxytocin. *J Neurosci* **30**:8096–8101.

García-Fernández JM, Álvarez-López C y Cernuda-Cernuda R (2007) Cytoplasmic localization of mPER1 clock protein isoforms in the mouse retina. *Neurosci Lett* **419**:55–58.

Gau P, Poon J, Ufret-Vincenty C, Snelson CD, Gordon SE, Raible DW y Dhaka A (2013) The zebrafish ortholog of TRPV1 is required for heat-induced locomotion. *J Neurosci* **33**:5249–5260.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS y Weitz CJ (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280**:1564–1569.

Gerhart-Hines Z, Feng D, Emmett MJ, Everett LJ, Loro E, Briggs ER, Bugge A, Hou C, Ferrara C, Seale P, Pryma DA, Khurana TS y Lazar MA (2013) The nuclear receptor Rev-erbα controls circadian thermogenic plasticity. *Nature* **503**:410–413.

Gervois P, Chopin-Delannoy S, Fadel A, Dubois G, Kosykh V, Fruchart J-C, Najïb J, Laudet V y Staels B (1999) Fibrates increase human REV-ERBα expression in liver via a novel peroxisome proliferator-activated receptor response element. *Mol Endocrinol* **13**:400–409.

Gillman AG, Rebec GV, Pecoraro NC y Kosobud AEK (2019) Circadian entrainment by food and drugs of abuse. *Behav Processes* **165**:23–28.

Gillum MP, Zhang D, Zhang X-M, Erion DM, Jamison RA, Choi C, Dong J, Shanabrough M, Duenas HR, Frederick DW, Hsiao JJ, Horvath TL, Lo CM, Tso P, Cline GW y Shulman GI (2008) N-acylphosphatidylethanolamine, a gut-derived circulating factor induced by fat ingestion, inhibits food intake. *Cell* **135**:813–824.

Girotti M, Weinberg MS y Spencer RL (2009) Diurnal expression of functional and clock-related genes throughout the rat HPA axis: system-wide shifts in response to a restricted feeding schedule. *Am J Physiol-Endocrinol Metab* **296**:E888–E897.

Glaser ST y Kaczocha M (2009) Temporal changes in mouse brain fatty acid amide hydrolase activity. *Neuroscience* **163**:594–600.

Godfrey N y Borgland SL (2019) Diversity in the lateral hypothalamic input to the ventral tegmental area. *Neuropharmacology* **154**:4–12.

Godlewski G, Offertáler L, Wagner JA y Kunos G (2009) Receptors for acylethanolamides—GPR55 and GPR119. *Prostaglandins Other Lipid Mediat* **89**:105–111.

Gómez-Boronat M, Velasco C, Isorna E, de Pedro N, Delgado MJ y Soengas JL (2016) The satiety factor oleoylethanolamide impacts hepatic lipid and glucose metabolism in goldfish. *J Comp Physiol B* **186**:1009–1021.

Gómez-Boronat M, Sáiz N, Delgado MJ, de Pedro N e Isorna E (2018) Time-lag in feeding schedule acts as a stressor that alters circadian oscillators in goldfish. *Front Physiol* **9**:1749.

Gómez-Boronat M, Isorna E, Armirotti A, Delgado MJ, Piomelli D y de Pedro N (2019) Diurnal profiles of N-acylethanolamines in goldfish brain y gastrointestinal tract: possible role of feeding. *Front Neurosci* **13**:450.

Gong N y Björnsson BT (2014) Leptin signaling in the rainbow trout central nervous system is modulated by a truncated leptin receptor isoform. *Endocrinology* **155**:2445–2455.

González-Yanes C, Serrano A, Bermúdez-Silva FJ, Hernández-Dominguez M, Páez-Ochoa MA, Rodríguez de Fonseca F y Sánchez-Margalet V (2005) Oleylethanolamide impairs glucose tolerance and inhibits insulin-stimulated glucose uptake in rat adipocytes through p38 and JNK MAPK pathways. *Am J Physiol-Endocrinol Metab* **289**:E923–E929.

Gorissen M y Flik G (2014) Leptin in teleostean fish, towards the origins of leptin physiology. *J Chem Neuroanat* **61–62**:200–206.

Grygiel-Górniak B (2014) Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications – a review. *Nutr J* **13**:17.

Guijarro A, Fu J, Astarita G y Piomelli D (2010) CD36 gene deletion decreases oleoylethanolamide levels in small intestine of free-feeding mice. *Pharmacol Res* **61**:27–33.

Guzmán M, LoVerme J, Fu J, Oveisi F, Blázquez C y Piomelli D (2004) Oleoylethanolamide stimulates lipolysis by activating the nuclear receptor peroxisome proliferator-activated receptor α (PPAR-α). *J Biol Chem* **279**:27849–27854.

H

Hansen HS (2010) Palmitoylethanolamide and other anandamide congeners. Proposed role in the diseased brain. *Exp Neurol* **224**:48–55.

Hansen HS (2013) Effect of diet on tissue levels of palmitoylethanolamide. *CNS Neurol Disord - Drug Targets* **12**:17–25.

Hansen HS (2014) Role of anorectic N-acylethanolamines in intestinal physiology and satiety control with respect to dietary fat. *Pharmacol Res* **86**:18–25.

Hansen HS y Diep TA (2009) N-acylethanolamines, anandamide and food intake. *Biochem Pharmacol* **78**:553–560.

Hansen HS y Vana V (2018) Non-endocannabinoid N-acylethanolamines and 2-monoacylglycerols in the intestine. *Br J Pharmacol* **176**:1443–1454.

Hara R, Wan K, Wakamatsu H, Aida R, Moriya T, Akiyama M y Shibata S (2001) Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. *Genes Cells* **6**:269–278.

Hastings M, O'Neill JS y Maywood ES (2007) Circadian clocks: regulators of endocrine and metabolic rhythms. *J Endocrinol* **195**:187–198.

Heisler LK y Lam DD (2017) An appetite for life: brain regulation of hunger and satiety. *Curr Opin Pharmacol* **37**:100–106.

Hernández-Pérez J, Míguez JM, Librán-Pérez M, Otero-Rodiño C, Naderi F, Soengas JL y López-Patiño MA (2015) Daily rhythms in activity and mRNA abundance of enzymes involved in glucose and lipid metabolism in liver of rainbow trout, *Oncorhynchus mykiss*. Influence of light and food availability. *Chronobiol Int* **32**:1391–1408.

Hernández-Pérez J, Míguez JM, Naderi F, Soengas JL y López-Patiño MA (2017) Influence of light and food on the circadian clock in liver of rainbow trout, *Oncorhynchus mykiss*. *Chronobiol Int* **34**:1259–1272.

Herrero MJ y Lepesant JMJ (2014) Daily and seasonal expression of clock genes in the pituitary of the European sea bass (*Dicentrarchus labrax*). *Gen Comp Endocrinol* **208**:30–38.

Hill MN, Miller GE, Carrier EJ, Gorzalka BB y Hillard CJ (2009) Circulating endocannabinoids y N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress. *Psychoneuroendocrinology* **34**:1257–1262.

Himick BA y Peter RE (1994a) Bombesin acts to suppress feeding behavior and alter serum growth hormone in goldfish. *Physiol Behav* **55**:65–72.

Himick BA y Peter RE (1994b) CCK/gastrin-like immunoreactivity in brain and gut, and CCK suppression of feeding in goldfish. *Am J Physiol-Regul Integr Comp Physiol* **267**:R841–R851.

Holubiec MI, Romero JI, Suárez J, Portavella M, Fernández-Espejo E, Blanco E, Galeano P y de Fonseca FR (2018) Palmitoylethanolamide prevents neuroinflammation, reduces astrogliosis and preserves recognition and spatial memory following induction of neonatal anoxia-ischemia. *Psychopharmacology (Berl)* **235**:2929–2945.

Hoskins LJ y Volkoff H (2012) The comparative endocrinology of feeding in fish: Insights and challenges. *Gen Comp Endocrinol* **176**:327–335.

Houston SJS, Karalazos V, Tinsley J, Betancor MB, Martin SAM, Tocher DR y Monroig O (2017) The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary fish oil and associated n-3 long-chain PUFA content. *Br J Nutr* **118**:1010–1022.

Hussain Z, Uyama T, Tsuboi K y Ueda N (2017) Mammalian enzymes responsible for the biosynthesis of N-acylethanolamines. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids* **1862**:1546–1561.

I

Iigo M y Tabata M (1996) Circadian rhythms of locomotor activity in the goldfish *Carassius auratus*. *Physiol Behav* **60**:775–781.

Inoue M, Tsuboi K, Okamoto Y, Hidaka M, Uyama T, Tsutsumi T, Tanaka T, Ueda N y Tokumura A (2017) Peripheral tissue levels and molecular species compositions of N-acyl-phosphatidylethanolamine and its metabolites in mice lacking N-acyl-phosphatidylethanolamine-specific phospholipase D. *J Biochem (Tokyo)* **162**:449–458.

Isorna E, Pedro N de, Valenciano AI, Alonso-Gómez ÁL y Delgado MJ (2017) Interplay between the endocrine and circadian systems in fishes. *J Endocrinol* **232**:R141–R159.

Izzo AA, Piscitelli F, Capasso R, Marini P, Cristino L, Petrosino S y Di Marzo V (2010) Basal and fasting/refeeding-regulated tissue levels of endogenous PPAR-α ligands in Zucker rats. *Obesity* **18**:55–62.

J

Jeong I, Kim E, Kim S, Kim H-K, Lee D-W, Seong JY y Park H-C (2018) mRNA expression and metabolic regulation of *npy* and *agrp1/2* in the zebrafish brain. *Neurosci Lett* **668**:73–79.

Ji W, Ping H-C, Wei K-J, Zhang G-R, Shi Z-C, Yang R-B, Zou G-W y Wang W-M (2015) Ghrelin, neuropeptide Y (NPY) and cholecystokinin (CCK) in blunt snout bream (*Megalobrama amblycephala*): cDNA cloning, tissue distribution and mRNA expression changes responding to fasting and refeeding. *Gen Comp Endocrinol* **223**:108–119.

Jönsson E (2013) The role of ghrelin in energy balance regulation in fish. *Gen Comp Endocrinol* **187**:79–85.

K

Kaeffer B y Pardini L (2005) Clock genes of mammalian cells: Practical implications in tissue culture. *Vitro Cell Dev Biol - Anim* **41**:311–320.

Kaiya H, Miyazato M, Kangawa K, Peter RE y Unniappan S (2008) Ghrelin: A multifunctional hormone in non-mammalian vertebrates. *Comp Biochem Physiol A Mol Integr Physiol* **149**:109–128.

Kakizawa T, Nishio S, Triqueneaux G, Bertrand S, Rambaud J y Laudet V (2007) Two differentially active alternative promoters control the expression of the zebrafish orphan nuclear receptor gene *Rev-erbα*. *J Mol Endocrinol* **38**:555–568.

Kang HS, Angers M, Beak JY, Wu X, Gimble JM, Wada T, Xie W, Collins JB, Grissom SF y Jetten AM (2007) Gene expression profiling reveals a regulatory role for RORα and RORγ in phase I and phase II metabolism. *Physiol Genomics* **31**:281–294.

Kang KS, Yahashi S y Matsuda K (2011) The effects of ghrelin on energy balance and psychomotor activity in a goldfish model: An overview. *Int J Pept* **2011**.

Kapoor BG, Smit H y Verighina IA (1976) The alimentary canal and digestion in teleosts. En: *Advances in Marine Biology* (Russell FS y Yonge M, eds), Academic Press, Londres, Reino Unido. pp 109–239.

Kasanen I, Inhilä K, Savontaus E, Voipio H-M, Õkva K, Mering S, Kiviniemi V, Hau J, Laaksonen S y Nevalainen T (2018) Diurnal rhythms of blood glucose, serum ghrelin, faecal IgA and faecal corticosterone in rats subjected to restricted feeding using the diet board. *Lab Anim* **52**:29–37.

Kaslin J, Nystedt JM, Östergård M, Peitsaro N y Panula P (2004) The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems. *J Neurosci* **24**:2678–2689.

Katsuyama Y, Oomiya Y, Dekimoto H, Motooka E, Takano A, Kikkawa S, Hibi M y Terashima T (2007) Expression of zebrafish *ROR alpha* gene in cerebellar-like structures. *Dev Dyn* **236**:2694–2701.

Kehoe AS y Volkoff H (2007) Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod (*Gadus morhua*). *Comp Biochem Physiol A Mol Integr Physiol* **146**:451–461.

Keppler D y Decker K (1974) Glycogen: Determination with amyloglucosidase. En: *Methods of enzimatic analysis* (Bergmeyer HU, ed), Academic Press, New York, NY, USA. pp 1127–1131.

Kerbel B y Unniappan S (2012) Nesfatin-1 suppresses energy intake, co-localises ghrelin in the brain and gut, and alters ghrelin, cholecystokinin and orexin mRNA expression in goldfish. *J Neuroendocrinol* **24**:366–377.

Kim M, de la Peña JB, Cheong JH y Kim HJ (2018) Neurobiological functions of the period circadian clock 2 gene, *per2*. *Biomol Ther* **26**:358–367.

Kitazawa T y Kaiya H (2019) Regulation of gastrointestinal motility by motilin and ghrelin in vertebrates. *Front Endocrinol* **10**:278.

Kleberg K, Hassing HA y Hansen HS (2014) Classical endocannabinoid-like compounds and their regulation by nutrients. *BioFactors* **40**:363–372.

Kobayashi Y, Ishikawa T, Hirayama J, Daiyasu H, Kanai S, Toh H, Fukuda I, Tsujimura T, Terada N, Kamei Y, Yuba S, Iwai S y Todo T (2000) Molecular analysis of zebrafish photolyase/cryptochrome family: two types of cryptochromes present in zebrafish. *Genes Cells* **5**:725–738.

Kojetin DJ y Burris TP (2014) REV-ERB and ROR nuclear receptors as drug targets. *Nat Rev Drug Discov* **13**:197–216.

Kolbe I, Brehm N y Oster H (2018) Interplay of central and peripheral circadian clocks in energy metabolism regulation. *J Neuroendocrinol* **31**:e12659.

Kopp R, Billecke N, Legradi J, Broeder M den, Parekh SH y Legler J (2016) Bringing obesity to light: Rev-erbα, a central player in light-induced adipogenesis in the zebrafish? *Int J Obes* **40**:824–832.

Kornmann B, Schaad O, Reinke H, Saini C y Schibler U (2007) Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators. *Cold Spring Harb Symp Quant Biol* **72**:319–330.

Kottelat M y Freyhof J (2007) *Handbook of European Freshwater Fishes*, Publications Kottelat, Cornol, Berlín, Alemania.

Koukkari WL y Sothern RB (2006) Chronobiometry: analyzing for rhythms. En: *Introducing biological rhythms* (Koukkari WL y Sothern RB, eds), Springer Science & Business Media, New York, NY, USA. pp 577–602.

Koven W y Schulte P (2012) The effect of fasting and refeeding on mRNA expression of PepT1 and gastrointestinal hormones regulating digestion and food intake in zebrafish (*Danio rerio*). *Fish Physiol Biochem* **38**:1565–1575.

Krug RG 2nd, Lee HB, Khoury LYE, Sigafoos AN, Petersen MO y Clark KJ (2018) The endocannabinoid gene *faah2a* modulates stress-associated behavior in zebrafish. *PLoS One* **13**:e0190897.

Kwon I, Choe HK, Son GH y Kim K (2011) Mammalian molecular clocks. *Exp Neurobiol* **20**:18–28.

Laitinen S, Fontaine C, Fruchart J y Staels B (2005) The role of the orphan nuclear receptor Rev-Erbα in adipocyte differentiation and function. *Biochimie* **87**:21–25.

Lamb CF (2001) Gustation and Feeding Behaviour. En: *Food Intake in Fish* (Houlihan D, Boujard T y Jobling M, eds), Blackwell Science, Oxford, Reino Unido. pp 108–130.

Lamia KA, Storch K-F y Weitz CJ (2008) Physiological significance of a peripheral tissue circadian clock. *Proc Natl Acad Sci U S A* **105**:15172–15177.

Langhans W (1999) Appetite regulation. En: *Protein metabolism y nutrition* (Lobley GE, White A y MacRae JC, eds), Wageningen Pers, Wageningen, Paises Bajos. pp 225–251.

Lau BK, Cota D, Cristino L y Borgland SL (2017) Endocannabinoid modulation of homeostatic and non-homeostatic feeding circuits. *Neuropharmacology* **124**:38–51.

Lauffer LM, Iakoubov R y Brubaker PL (2009) GPR119 is essential for oleoylethanolamide-induced glucagon-like peptide-1 secretion from the intestinal enteroendocrine L-cell. *Diabetes* **58**:1058–1066.

Lazado CC, Kumaratunga HPS, Nagasawa K, Babiak I, Giannetto A y Fernandes JMO (2014) Daily rhythmicity of clock gene transcripts in Atlantic cod fast skeletal muscle. *PLoS One* **9**:e99172.

Leal E, Fernández-Durán B, Agulleiro MJ, Conde-Siera M, Míguez JM y Cerdá-Reverter JM (2013) Effects of dopaminergic system activation on feeding behavior and growth performance of the sea bass (*Dicentrarchus labrax*): A self-feeding approach. *Horm Behav* **64**:113–121.

Lecarpentier Y, Claes V, Duthoit G y Hébert J-L (2014) Circadian rhythms, Wnt/beta-catenin pathway and PPAR alpha/gamma profiles in diseases with primary or secondary cardiac dysfunction. *Front Physiol* **5**:429.

Leishman E, Mackie K, Luquet S y Bradshaw HB (2016) Lipidomics profile of a NAPE-PLD KO mouse provides evidence of a broader role of this enzyme in lipid metabolism in the brain. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids* **1861**:491–500.

Leproult R, Holmbäck U y Cauter EV (2014) Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss. *Diabetes* **63**:1860–1869.

Li M, Wang D, Bi W, Jiang Z, Piao R y Yu H (2019) N-Palmitoylethanolamide exerts antidepressant-like effects in rats: Involvement of PPARα pathway in the hippocampus. *J Pharmacol Exp Ther* **369**:163–172.

Librán-Pérez M, Polakof S, López-Patiño MA, Míguez JM y Soengas JL (2012) Evidence of a metabolic fatty acid-sensing system in the hypothalamus and Brockmann bodies of rainbow trout: implications in food intake regulation. *Am J Physiol-Regul Integr Comp Physiol* **302**:R1340–R1350.

Librán-Pérez M, Figueiredo-Silva AC, Panserat S, Geurden I, Míguez JM, Polakof S y Soengas JL (2013a) Response of hepatic lipid and glucose metabolism to a mixture or single fatty acids: Possible presence of fatty acid-sensing mechanisms. *Comp Biochem Physiol A Mol Integr Physiol* **164**:241–248.

Librán-Pérez M, López-Patiño MA, Míguez JM y Soengas JL (2013b) *In vitro* response of putative fatty acid-sensing systems in rainbow trout liver to increased levels of oleate or octanoate. *Comp Biochem Physiol A Mol Integr Physiol* **165**:288–294.

Librán-Pérez M, Otero-Rodiño C, López-Patiño MA, Míguez JM y Soengas JL (2014) Central administration of oleate or octanoate activates hypothalamic fatty acid sensing and inhibits food intake in rainbow trout. *Physiol Behav* **129**:272–279.

Librán-Pérez M, Geurden I, Dias K, Corraze G, Panserat S y Soengas JL (2015a) Feeding rainbow trout with a lipid-enriched diet: effects on fatty acid sensing, regulation of food intake and cellular signaling pathways. *J Exp Biol* **218**:2610–2619.

Librán-Pérez M, Velasco C, Otero-Rodiño C, López-Patiño MA, Míguez M Jesús M y Soengas S José L (2015b) Effects of insulin treatment on the response to oleate and octanoate of

food intake and fatty acid–sensing systems in rainbow trout. *Domest Anim Endocrinol* **53**:124–135.

Liedhegner ES, Sasman A y Hillard CJ (2014) Brain region-specific changes in N-acylethanolamine contents with time of day. *J Neurochem* **128**:491–506.

Lin L, Metherel AH, Kitson AP, Alashmali SM, Hopperton KE, Trépanier M-O, Jones PJ y Bazinet RP (2018) Dietary fatty acids augment tissue levels of n-acylethanolamines in n-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) knockout mice. *J Nutr Biochem* **62**:134–142.

Liu S, Alexander RK y Lee C-H (2014) Lipid metabolites as metabolic messengers in inter-organ communication. *Trends Endocrinol Metab* **25**:356–363.

Livak KJ y Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. *Methods* **25**:402–408.

Londraville RL, Macotela Y, Duff RJ, Easterling MR, Liu Q y Crespi EJ (2014) Comparative endocrinology of leptin: Assessing function in a phylogenetic context. *Gen Comp Endocrinol* **203**:146–157.

López-Olmeda JF (2017) Nonphotic entrainment in fish. *Comp Biochem Physiol A Mol Integr Physiol* **203**:133–143.

López-Olmeda JF, Montoya A, Oliveira C y Sánchez-Vázquez FJ (2009) Synchronization to light and restricted-feeding schedules of behavioral and humoral daily rhythms in gilthead sea bream (*Sparus aurata*). *Chronobiol Int* **26**:1389–1408.

López-Olmeda JF, Tartaglione EV, de la Iglesia HO y Sánchez-Vázquez FJ (2010) Feeding entrainment of food-anticipatory activity and *per1* expression in the brain and liver of zebrafish under different lighting and feeding conditions. *Chronobiol Int* **27**:1380–1400.

López-Patiño MA, Guijarro AI, Isorna E, Delgado MJ, Alonso-Bedate M y de Pedro N (1999) Neuropeptide Y has a stimulatory action on feeding behavior in goldfish (*Carassius auratus*). *Eur J Pharmacol* **377**:147–153.

LoVerme J, Fu J, Astarita G, Rana GL, Russo R, Calignano A y Piomelli D (2005) The nuclear receptor peroxisome proliferator-activated receptor-α mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol* **67**:15–19.

Lu Y, Reyes J, Walter S, Gonzalez T, Medrano G, Boswell M, Boswell W, Savage M y Walter R (2018) Characterization of basal gene expression trends over a diurnal cycle in *Xiphophorus maculatus* skin, brain and liver. *Comp Biochem Physiol Toxicol Pharmacol CBP* **208**:2–11.

MacDonald E y Volkoff H (2009a) Cloning, distribution y effects of season y nutritional status on the expression of neuropeptide Y (NPY), cocaine and amphetamine regulated transcript (CART) and cholecystokinin (CCK) in winter flounder (*Pseudopleuronectes americanus*). *Horm Behav* **56**:58–65.

MacDonald E y Volkoff H (2009b) Neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART) and cholecystokinin (CCK) in winter skate (*Raja ocellata*): cDNA cloning, tissue distribution and mRNA expression responses to fasting. *Gen Comp Endocrinol* **161**:252–261.

Madrid JA (2006) Los relojes de la vida. Una introducción a la Cronobiología. En: *Cronobiología básica y clínica* (Madrid Pérez JA y Rol de Lama Á, eds), Editec @Red, Madrid, España. pp 39–81.

Maradonna F y Carnevali O (2018) Lipid metabolism alteration by endocrine disruptors in animal models: An overview. *Front Endocrinol* **9**:654.

Marcheva B, Ramsey KM, Affinati A y Bass J (2009) Clock genes and metabolic disease. *J Appl Physiol* **107**:1638–1646.

Martella A, Silvestri C, Maradonna F, Gioacchini G, Allarà M, Radaelli G, Overby DR, Di Marzo V y Carnevali O (2016) Bisphenol A induces fatty liver by an endocannabinoid-mediated positive feedback loop. *Endocrinology* **157**:1751–1763.

Martín-Robles ÁJ, Whitmore D, Sánchez-Vázquez FJ, Pendón C y Muñoz-Cueto JA (2012) Cloning, tissue expression pattern and daily rhythms of *Period1*, *Period2*, and *Clock* transcripts in the flatfish Senegalese sole, *Solea senegalensis*. *J Comp Physiol B* **182**:673–685.

Matias I, Gonthier M-P, Petrosino S, Docimo L, Capasso R, Hoareau L, Monteleone P, Roche R, Izzo AA y Di Marzo V (2007) Role and regulation of acylethanolamides in energy balance: focus on adipocytes and β-cells. *Br J Pharmacol* **152**:676–690.

Matsuda K, Miura T, Kaiya H, Maruyama K, Shimakura S-I, Uchiyama M, Kangawa K y Shioda S (2006) Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. *Peptides* **27**:2321–2325.

Matsuda K, Shimakura S-I, Miura T, Maruyama K, Uchiyama M, Kawauchi H, Shioda S y Takahashi A (2007) Feeding-induced changes of melanin-concentrating hormone (MCH)-like immunoreactivity in goldfish brain. *Cell Tissue Res* **328**:375–382.

Matsuda K, Sakashita A, Yokobori E y Azuma M (2012) Neuroendocrine control of feeding behavior and psychomotor activity by neuropeptide Y in fish. *Neuropeptides* **46**:275–283.

Mattace Raso G, Russo R, Calignano A y Meli R (2014a) Palmitoylethanolamide in CNS health and disease. *Pharmacol Res* **86**:32–41.

Mattace Raso G, Santoro A, Russo R, Simeoli R, Paciello O, Di Carlo C, Diano S, Calignano A y Meli R (2014b) Palmitoylethanolamide prevents metabolic alterations and restores leptin sensitivity in ovariectomized rats. *Endocrinology* **155**:1291–1301.

Mazzola C, Medalie J, Scherma M, Panlilio LV, Solinas M, Tanda G, Drago F, Cadet JL, Goldberg SR y Yasar S (2009) Fatty acid amide hydrolase (FAAH) inhibition enhances memory acquisition through activation of PPAR-α nuclear receptors. *Learn Mem* **16**:332–337.

McPartland JM, Glass M, Matias I, Norris RW y Kilpatrick CW (2007) A shifted repertoire of endocannabinoid genes in the zebrafish (*Danio rerio*). *Mol Genet Genomics* **277**:555–570.

McVay JA y Kaan HW (1940) The digestive tract of *Carassius auratus*. *Biol Bull* **78**:53–67.

Mehra A, Baker CL, Loros JJ y Dunlap JC (2009) Post-translational modifications in circadian rhythms. *Trends Biochem Sci* **34**:483–490.

Meier AH (1976) Daily variation in concentration of plasma corticosteroid in hypophysectomized rats. *Endocrinology* **98**:1475–1479.

Mendoza J y Challet E (2009) Brain clocks: from the suprachiasmatic nuclei to a cerebral network. *Neurosci Rev J Bringing Neurobiol Neurol Psychiatry* **15**:477–488.

Metz JR, Peters JJM y Flik G (2006) Molecular biology and physiology of the melanocortin system in fish: A review. *Gen Comp Endocrinol* **148**:150–162.

Mimeault C, Trudeau VL y Moon TW (2006) Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARβ) mRNA levels in male goldfish (*Carassius auratus*). *Toxicology* **228**:140–150.

Miura T, Maruyama K, Shimakura S-I, Kaiya H, Uchiyama M, Kangawa K, Shioda S y Matsuda K (2006) Neuropeptide Y mediates ghrelin-induced feeding in the goldfish, *Carassius auratus*. *Neurosci Lett* **407**:279–283.

Miura T, Maruyama K, Shimakura S-I, Kaiya H, Uchiyama M, Kangawa K, Shioda S y Matsuda K (2007) Regulation of food intake in the goldfish by interaction between ghrelin and orexin. *Peptides* **28**:1207–1213.

Monteleone AM, Di Marzo V, Monteleone P, Dalle Grave R, Aveta T, Ghoch ME, Piscitelli F, Volpe U, Calugi S y Maj M (2016) Responses of peripheral endocannabinoids and endocannabinoid-related compounds to hedonic eating in obesity. *Eur J Nutr* **55**:1799–1805.

Montoya A, López-Olmeda JF, Garayzar ABS y Sánchez-Vázquez FJ (2010) Synchronization of daily rhythms of locomotor activity and plasma glucose, cortisol and thyroid hormones to feeding in Gilthead seabream (*Sparus aurata*) under a light–dark cycle. *Physiol Behav* **101**:101–107.

Moore HA y Whitmore D (2014) Circadian rhythmicity and light sensitivity of the zebrafish brain. *PLoS ONE* **9**:e86176.

Morton GJ, Cummings DE, Baskin DG, Barsh GS y Schwartz MW (2006) Central nervous system control of food intake and body weight. *Nature* **443**:289–295.

Mühlbauer E, Wolgast S, Finckh U, Peschke D y Peschke E (2004) Indication of circadian oscillations in the rat pancreas. *FEBS Lett* **564**:91–96.

Mukherji A, Kobiita A y Chambon P (2015) Shifting the feeding of mice to the rest phase creates metabolic alterations, which, on their own, shift the peripheral circadian clocks by 12 hours. *Proc Natl Acad Sci* **112**:E6683–E6690.

Murashita K, Fukada H, Hosokawa H y Masumoto T (2006) Cholecystokinin y peptide Y in yellowtail (*Seriola quinqueradiata*): Molecular cloning, real-time quantitative RT-PCR, and response to feeding y fasting. *Gen Comp Endocrinol* **145**:287–297.

Murashita K, Uji S, Yamamoto T, Rønnestad I y Kurokawa T (2008) Production of recombinant leptin and its effects on food intake in rainbow trout (*Oncorhynchus mykiss*). *Comp Biochem Physiol B Biochem Mol Biol* **150**:377–384.

Murillo-Rodriguez E, Désarnaud F y Prospéro-García O (2006) Diurnal variation of arachidonoylethanolamine, palmitoylethanolamide and oleoylethanolamide in the brain of the rat. *Life Sci* **79**:30–37.

Muus BJ y Dahlström P (1981) *Los peces de agua dulce de España y de Europa: pesca, biología, importancia económica*, Omega, Barcelona, España.

N

Nader N, Chrousos GP y Kino T (2010) Interactions of the circadian CLOCK system and the HPA axis. *Trends Endocrinol Metab TEM* **21**:277–286.

Nakamachi T, Matsuda K, Maruyama K, Miura T, Uchiyama M, Funahashi H, Sakurai T y Shioda S (2006) Regulation by orexin of feeding behaviour and locomotor activity in the goldfish. *J Neuroendocrinol* **18**:290–297.

Nakamura K, Inoue I, Takahashi S, Komoda T y Katayama S (2008a) Cryptochrome and period proteins are regulated by the CLOCK/BMAL1 gene: Crosstalk between the PPARs/RXRα-regulated y CLOCK/BMAL1-regulated systems. *PPAR Res* **2008**:348610.

Narnaware YK y Peter RE (2001) Effects of food deprivation and refeeding on neuropeptide Y (NPY) mRNA levels in goldfish. *Comp Biochem Physiol B Biochem Mol Biol* **129**:633–637.

Narnaware YK y Peter RE (2002) Influence of diet composition on food intake and neuropeptide Y (NPY) gene expression in goldfish brain. *Regul Pept* **103**:75–83.

Narnaware YK, Peyon PP, Lin X y Peter RE (2000) Regulation of food intake by neuropeptide Y in goldfish. *Am J Physiol-Regul Integr Comp Physiol* **279**:R1025–R1034.

Nisembaum LG, Velarde E, Tinoco AB, Azpeleta C, de Pedro N, Alonso-Gómez AL, Delgado MJ e Isorna E (2012) Light-dark cycle and feeding time differentially entrains the gut molecular clock of the goldfish (*Carassius auratus*). *Chronobiol Int* **29**:665–673.

Nisembaum LG, de Pedro N, Delgado MJ, Sánchez-Bretaño A e Isorna E (2014) Orexin as an input of circadian system in goldfish: Effects on clock gene expression and locomotor activity rhythms. *Peptides* **52**:29–37.

Nogueiras R, Romero-Picó A, Vazquez MJ, Novelle MG, López M y Diéguez C (2012) The opioid system and food intake: Homeostatic y hedonic mechanisms. *Obes Facts* **5**:196–207.

Nuclear Receptors Nomenclature Committee (1999) A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97**:161–163.

O

Oishi K, Atsumi G, Sugiyama S, Kodomari I, Kasamatsu M, Machida K y Ishida N (2006) Disrupted fat absorption attenuates obesity induced by a high-fat diet in *Clock* mutant mice. *FEBS Lett* **580**:127–130.

Okamoto Y, Morishita J, Tsuboi K, Tonai T y Ueda N (2004) Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem* **279**:5298–5305.

Opazo R, Plaza-Parrochia F, Cardoso dos Santos GR, Carneiro GRA, Sardela VF, Romero J y Valladares L (2019) Fasting upregulates *npy*, *agrp*, and *ghsr* without increasing ghrelin levels in zebrafish (*Danio rerio*) larvae. *Front Physiol* **9**:1901.

Oster H, Damerow S, Kiessling S, Jakubcakova V, Abraham D, Tian J, Hoffmann MW y Eichele G (2006) The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metab* **4**:163–173.

Otero-Rodiño C, Librán-Pérez M, Velasco C, López-Patiño MA, Míguez JM y Soengas JL (2015) Evidence for the presence of glucosensor mechanisms not dependent on glucokinase in hypothalamus and hindbrain of rainbow trout (*Oncorhynchus mykiss*). *PLoS One* **10**:e0128603.

Otero-Rodiño C, Velasco C, Álvarez-Otero R, López-Patiño MA, Míguez JM y Soengas JL (2016) *In vitro* evidence in rainbow trout supporting glucosensing mediated by sweet taste receptor, LXR, and mitochondrial activity in Brockmann bodies, and sweet taste receptor in liver. *Comp Biochem Physiol B Biochem Mol Biol* **200**:6–16.

P

Panda S (2016) Circadian physiology of metabolism. *Science* **354**:1008–1015.

Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS y Hogenesch JB (2002a) Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* **109**:307–320.

Panda S, Hogenesch JB y Kay SA (2002b) Circadian rhythms from flies to human. *Nature* **417**:329–335.

Paredes JF, Vera LM, Martinez-Lopez FJ, Navarro I y Vázquez FJS (2014) Circadian rhythms of gene expression of lipid metabolism in Gilthead sea bream liver: Synchronisation to light and feeding time. *Chronobiol Int* **31**:613–626.

Paredes JF, López-Olmeda JF, Martínez FJ y Sánchez-Vázquez FJ (2015) Daily rhythms of lipid metabolic gene expression in zebra fish liver: Response to light/dark y feeding cycles. *Chronobiol Int* **32**:1438–1448.

Partch CL, Green CB y Takahashi JS (2014) Molecular architecture of the mammalian circadian clock. *Trends Cell Biol* **24**:90–99.

Patton DF y Mistlberger RE (2013) Circadian adaptations to meal timing: neuroendocrine mechanisms. *Front Neurosci* **7**:185.

Pavón FJ, Serrano A, Romero-Cuevas M, Alonso M y Rodríguez de Fonseca F (2010) Oleoylethanolamide: a new player in peripheral control of energy metabolism. Therapeutic implications. *Drug Discov Today Dis Mech* **7**:e175–e183.

Pawlak M, Lefebvre P y Staels B (2015) Molecular mechanism of PPARα action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. *J Hepatol* **62**:720–733.

Peirson SN, Butler JN, Duffield GE, Takher S, Sharma P y Foster RG (2006) Comparison of clock gene expression in SCN, retina, heart, and liver of mice. *Biochem Biophys Res Commun* **351**:800–807.

Peng H, Zhu Y, Goldberg J, Vaccarino V y Zhao J (2019) DNA methylation of five core circadian genes jointly contributes to glucose metabolism: A gene-set analysis in monozygotic twins. *Front Genet* **10**:329.

Petersen G, Sørensen C, Schmid PC, Artmann A, Tang-Christensen M, Hansen SH, Larsen PJ, Schmid HHO y Hansen HS (2006) Intestinal levels of anandamide and oleoylethanolamide in food-deprived rats are regulated through their precursors. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids* **1761**:143–150.

Petrosino S y Di Marzo V (2017) The pharmacology of palmitoylethanolamide and first data on the therapeutic efficacy of some of its new formulations. *Br J Pharmacol* **174**:1349–1365.

Pett JP, Kondoff M, Bordyugov G, Kramer A y Herzel H (2018) Co-existing feedback loops generate tissue-specific circadian rhythms. *Life Sci Alliance* **1**:e201800078.

Piccinetti CC, Migliarini B, Petrosino S, Di Marzo V y Carnevali O (2010) Anandamide and AM251, via water, modulate food intake at central and peripheral level in fish. *Gen Comp Endocrinol* **166**:259–267.

Piomelli D (2013) A fatty gut feeling. *Trends Endocrinol Metab* **24**:332–341.

Pittendrigh CS (1993) Temporal organization: reflections of a Darwinian clock-watcher. *Annu Rev Physiol* **55**:17–54.

Polakof S, Míguez JM, Moon TW y Soengas JL (2007a) Evidence for the presence of a glucosensor in hypothalamus, hindbrain, and Brockmann bodies of rainbow trout. *Am J Physiol-Regul Integr Comp Physiol* **292**:R1657–R1666.

Polakof S, Míguez JM y Soengas JL (2007b) *In vitro* evidences for glucosensing capacity y mechanisms in hypothalamus, hindbrain, and Brockmann bodies of rainbow trout. *Am J Physiol-Regul Integr Comp Physiol* **293**:R1410–R1420.

Polakof S, Míguez JM y Soengas JL (2008a) Changes in food intake y glucosensing function of hypothalamus and hindbrain in rainbow trout subjected to hyperglycemic or hypoglycemic conditions. *J Comp Physiol A* **194**:829–839.

Polakof S, Míguez JM y Soengas JL (2008b) Dietary carbohydrates induce changes in glucosensing capacity and food intake of rainbow trout. *Am J Physiol-Regul Integr Comp Physiol* **295**:R478–R489.

Polakof S, Panserat S, Plagnes-Juan E y Soengas JL (2008c) Altered dietary carbohydrates significantly affect gene expression of the major glucosensing components in Brockmann bodies and hypothalamus of rainbow trout. *Am J Physiol-Regul Integr Comp Physiol* **295**:R1077–R1088.

Polakof S, Médale F, Skiba-Cassy S, Corraze G y Panserat S (2010) Molecular regulation of lipid metabolism in liver and muscle of rainbow trout subjected to acute y chronic insulin treatments. *Domest Anim Endocrinol* **39**:26–33.

Polakof S, Panserat S, Soengas JL y Moon TW (2012) Glucose metabolism in fish: a review. *J Comp Physiol B* **182**:1015–1045.

Preitner N, Damiola F, Luis-Lopez-Molina, Zakany J, Duboule D, Albrecht U y Schibler U (2002) The orphan nuclear receptor REV-ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**:251–260.

Proulx K, Cota D, Castañeda TR, Tschöp MH, D'Alessio DA, Tso P, Woods SC y Seeley RJ (2005) Mechanisms of oleoylethanolamide-induced changes in feeding behavior and motor activity. *Am J Physiol-Regul Integr Comp Physiol* **289**:R729–R737.

Q

Qian J y Scheer FA (2016) Circadian system and glucose metabolism: implications for physiology y disease. *Trends Endocrinol Metab* **27**:282–293.

R

Rahman IAS, Tsuboi K, Uyama T y Ueda N (2014) New players in the fatty acyl ethanolamide metabolism. *Pharmacol Res* **86**:1–10.

Rakhshandehroo M, Knoch B, Müller M y Kersten S (2010) Peroxisome proliferator-activated receptor alpha target genes. *PPAR Res* **2010**.

Ramirez-Plascencia OD, Saderi N, Escobar C y Salgado-Delgado RC (2017) Feeding during the rest phase promotes circadian conflict in nuclei that control energy homeostasis and sleep–wake cycle in rats. *Eur J Neurosci* **45**:1325–1332.

Rao MN, Neylan TC, Grunfeld C, Mulligan K, Schambelan M y Schwarz J-M (2015) Subchronic sleep restriction causes tissue-specific insulin resistance. *J Clin Endocrinol Metab* **100**:1664–1671.

Raspè E, Mautino G, Duval C, Fontaine C, Duez H, Barbier O, Monte D, Fruchart J, Fruchart J-C y Staels B (2002) Transcriptional regulation of human rev-erbα gene expression by the orphan nuclear receptor retinoic acid-related orphan receptor α. *J Biol Chem* **277**:49275–49281.

Refinetti R (2006) *Circadian physiology*, 2nd ed, CRC Press/Taylor & Francis Group, Boca Ratón, FL, USA.

Refinetti R (2015) Comparison of light, food and temperature as environmental synchronizers of the circadian rhythm of activity in mice. *J Physiol Sci* **65**:359–366.

Reinke H y Asher G (2016) Circadian clock control of liver metabolic functions. *Gastroenterology* **150**:574–580.

Reinke H y Asher G (2019) Crosstalk between metabolism and circadian clocks. *Nat Rev Mol Cell Biol* **20**:227–241.

Reppert SM y Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* **418**:935–941.

Ribas-Latre A y Eckel-Mahan K (2016) Interdependence of nutrient metabolism and the circadian clock system: Importance for metabolic health. *Mol Metab* **5**:133–152.

Rodríguez de Fonseca F, Navarro M, Gómez R, Escuredo L, Nava F, Fu J, Murillo-Rodríguez E, Giuffrida A, LoVerme J, Gaetani S, Kathuria S, Gall C y Piomelli D (2001) An anorexic lipid mediator regulated by feeding. *Nature* **414**:209–212.

Romano A, Potes CS, Tempesta B, Cassano T, Cuomo V, Lutz T y Gaetani S (2013) Hindbrain noradrenergic input to the hypothalamic PVN mediates the activation of oxytocinergic neurons induced by the satiety factor oleoylethanolamide. *Am J Physiol-Endocrinol Metab* **305**:E1266–E1273.

Romano A, Tempesta B, Provensi G, Passani MB y Gaetani S (2015) Central mechanisms mediating the hypophagic effects of oleoylethanolamide and N-acylphosphatidylethanolamines: different lipid signals? *Front Pharmacol* **6**:137.

Rønnestad I, Gomes AS, Murashita K, Angotzi R, Jönsson E y Volkoff H (2017) Appetite-controlling endocrine systems in teleosts. *Front Endocrinol* **8**:73.

Rudic RD, McNamara P, Reilly D, Grosser T, Curtis A-M, Price TS, Panda S, Hogenesch JB y FitzGerald GA (2005) Bioinformatic analysis of circadian gene oscillation in mouse aorta. *Circulation* **112**:2716–2724.

Rui L (2013) Brain regulation of energy balance and body weight. *Rev Endocr Metab Disord* **14**:387–407.

Rui L (2014) Energy metabolism in the liver. *Compr Physiol* **4**:177–197.

Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson N-O, Leonova J, Elebring T, Nilsson K, Drmota T y Greasley PJ (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol* **152**:1092–1101.

S

Saitou N y Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**:406–425.

Sam AH, Troke RC, Tan TM y Bewick GA (2012) The role of the gut/brain axis in modulating food intake. *Neuropharmacology* **63**:46–56.

Sanchez JA y Sanchez-Vazquez FJ (2009) Feeding entrainment of daily rhythms of locomotor activity and clock gene expression in zebrafish brain. *Chronobiol Int* **26**:1120–1135.

Sánchez JA, Madrid JA y Sánchez-Vázquez FJ (2010) Molecular cloning, tissue distribution, and daily rhythms of expression of *per1* gene in European sea bass (*Dicentrarchus labrax*). *Chronobiol Int* **27**:19–33.

Sánchez-Bretaño A, Alonso-Gómez ÁL, Delgado MJ e Isorna E (2015a) The liver of goldfish as a component of the circadian system: Integrating a network of signals. *Gen Comp Endocrinol* **221**:213–216.

Sánchez-Bretaño A, Gueguen M-M, Cano-Nicolau J, Kah O, Alonso-Gómez ÁL, Delgado MJ e Isorna E (2015b) Anatomical distribution and daily profile of *gper1b* gene expression in brain and peripheral structures of goldfish (*Carassius auratus*). *Chronobiol Int* **32**:889–902.

Sánchez-Bretaño A, Callejo M, Montero M, Alonso-Gómez ÁL, Delgado MJ e Isorna E (2016) Performing a hepatic timing signal: glucocorticoids induce *gper1a* and *gper1b* expression and repress *gclock1a* and *bmal1a* in the liver of goldfish. *J Comp Physiol B* **186**:73–82.

Sánchez-Gurmaches J, Cruz-Garcia L, Gutiérrez J y Navarro I (2012) Adiponectin effects and gene expression in rainbow trout: an *in vivo* and *in vitro* approach. *J Exp Biol* **215**:1373–1383.

Sánchez-Vázquez FJ, Madrid JA, Zamora S, Iigo M y Tabata M (1996) Demand feeding and locomotor circadian rhythms in the goldfish, *Carassius auratus*: Dual and independent phasing. *Physiol Behav* **60**:665–674.

Sarbahi DS (1951) Studies of the digestive tracts and the digestive enzymes of the goldfish, *Carassius auratus* (Linnaeus) and the largemouth black bass, *Micropterus salmoides* (Lacépède). *Biol Bull* **100**:244–257.

Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA y Hogenesch JB (2004) A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron* **43**:527–537.

Schaaf MJM (2017) Nuclear receptor research in zebrafish. *J Mol Endocrinol* **59**:R65–R76.

Schibler U (2006) Circadian time keeping: the daily ups and downs of genes, cells, and organisms. En: *Progress in Brain Research* (Kalsbeek A, Fliers E, Hofman MA, Swaab DF, van Someren EJW y Buijs RM, eds), Elsevier, Amsterdam, Paises Bajos. pp 271–282.

Schibler U, Ripperger J y Brown SA (2003) Peripheral circadian oscillators in mammals: Time and food. *J Biol Rhythms* **18**:250–260.

Schjolden J, Schiöth HB, Larhammar D, Winberg S y Larson ET (2009) Melanocortin peptides affect the motivation to feed in rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* **160**:134–138.

Schibler U, Gotic I, Saini C, Gos P, Curie T, Emmenegger Y, Sinturel F, Gosselin P, Gerber A, Fleury-Olela F, Rando G, Demarque M y Franken P (2015) Clock-talk: Interactions between central and peripheral circadian oscillators in mammals. *Cold Spring Harb Symp Quant Biol* **80**:223–232.

Schmutz I, Albrecht U y Ripperger JA (2012) The role of clock genes and rhythmicity in the liver. *Mol Cell Endocrinol* **349**:38–44.

Schreck CB y Tort L (2016) The concept of stress in fish. En: *Fish Physiology* (Schreck CB, Tort L, Farrell AP y Brauner CJ, eds), Academic Press, Londres, Reino Unido. pp 1–34.

Schwartz GJ, Fu J, Astarita G, Li X, Gaetani S, Campolongo P, Cuomo V y Piomelli D (2008) The lipid messenger OEA links dietary fat intake to satiety. *Cell Metab* **8**:281–288.

Serrano A, del Arco I, Javier Pavón F, Macías M, Perez-Valero V y Rodríguez de Fonseca F (2008) The cannabinoid CB1 receptor antagonist SR141716A (Rimonabant) enhances the metabolic benefits of long-term treatment with oleoylethanolamide in Zucker rats. *Neuropharmacology* **54**:226–234.

Serrano A, Pavón FJ, Tovar S, Casanueva F, Señarís R, Diéguez C y Rodríguez de Fonseca F (2011) Oleoylethanolamide: Effects on hypothalamic transmitters and gut peptides regulating food intake. *Neuropharmacology* **60**:593–601.

Shimba S, Ishii N, Ohta Y, Ohno T, Watabe Y, Hayashi M, Wada T, Aoyagi T y Tezuka M (2005) Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. *Proc Natl Acad Sci U S A* **102**:12071–12076.

Shimba S, Ogawa T, Hitosugi S, Ichihashi Y, Nakadaira Y, Kobayashi M, Tezuka M, Kosuge Y, Ishige K, Ito Y, Komiyama K, Okamatsu-Ogura Y, Kimura K y Saito M (2011) Deficient of a clock gene, brain and muscle arnt-like protein-1 (BMAL1), induces dyslipidemia and ectopic fat formation. *PLoS One* **6**:e25231.

Sihag J y Jones PJH (2018) Oleoylethanolamide: The role of a bioactive lipid amide in modulating eating behaviour. *Obes Rev* **19**:178–197.

Silverstein JT y Plisetskaya EM (2000) The effects of NPY and insulin on food intake regulation in fish. *Am Zool* **40**:296–308.

Skaper SD, Facci L, Barbierato M, Zusso M, Bruschetta G, Impellizzeri D, Cuzzocrea S y Giusti P (2015) N-palmitoylethanolamine and neuroinflammation: a novel therapeutic strategy of resolution. *Mol Neurobiol* **52**:1034–1042.

Sládek M, Rybová M, Jindráková Z, Zemanová Z, Polidarová L, Mrnka L, O'Neill J, Pácha J y Sumová A (2007) Insight into the circadian clock within rat colonic epithelial cells. *Gastroenterology* **133**:1240–1249.

Sobrino Crespo C, Perianes Cachero A, Puebla Jiménez L, Barrios V y Arilla Ferreiro E (2014) Peptides and food intake. *Front Endocrinol* **5**:58.

Soengas JL (2014) Contribution of glucose- and fatty acid sensing systems to the regulation of food intake in fish. A review. *Gen Comp Endocrinol* **205**:36–48.

Soengas JL y Aldegunde M (2004) Brain glucose and insulin: effects on food intake and brain biogenic amines of rainbow trout. *J Comp Physiol A* **190**:641–649.

Soengas JL, Cerdá-Reverter JM y Delgado MJ (2018) Central regulation of food intake in fish: an evolutionary perspective. *J Mol Endocrinol* **60**:R171–R199.

Sohn J-W (2015) Network of hypothalamic neurons that control appetite. *BMB Rep* **48**:229–233.

Solt LA, Kojetin DJ y Burris TP (2011) The REV-ERBs and RORs: molecular links between circadian rhythms and lipid homeostasis. *Future Med Chem* **3**:623–638.

Son GH, Chung S, Choe HK, Kim H-D, Baik S-M, Lee H, Lee H-W, Choi S, Sun W, Kim H, Cho S, Lee KH y Kim K (2008) Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proc Natl Acad Sci* **105**:20970–20975.

Spencer RL, Chun LE, Hartsock MJ y Woodruff ER (2018) Glucocorticoid hormones are both a major circadian signal and major stress signal: How this shared signal contributes to a dynamic relationship between the circadian and stress systems. *Front Neuroendocrinol* **49**:52–71.

Srivastava AK y Meier AH (1972) Daily variation in concentration of cortisol in plasma in intact and hypophysectomized gulf killifish. *Science* **177**:185–187.

Stephan FK (2002) The "other" circadian system: Food as a zeitgeber. *J Biol Rhythms* **17**:284–292.

Stokkan K-A, Yamazaki S, Tei H, Sakaki Y y Menaker M (2001) Entrainment of the circadian clock in the liver by feeding. *Science* **291**:490–493.

Storch K-F, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH y Weitz CJ (2002) Extensive and divergent circadian gene expression in liver and heart. *Nature* **417**:78–83.

Suárez J, Rivera P, Arrabal S, Crespillo A, Serrano A, Baixeras E, Pavón FJ, Cifuentes M, Nogueiras R, Ballesteros J, Dieguez C y Rodríguez de Fonseca F (2014)

Oleoylethanolamide enhances β-adrenergic-mediated thermogenesis and white-to-brown adipocyte phenotype in epididymal white adipose tissue in rat. *Dis Model Mech* **7**:129–141.

T

Tahara Y y Shibata S (2018) Entrainment of the mouse circadian clock: Effects of stress, exercise, and nutrition. *Free Radic Biol Med* **119**:129–138.

Tamura K, Stecher G, Peterson D, Filipski A y Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* **30**:2725–2729.

Terrazzino S, Berto F, Carbonare MD, Fabris M, Guiotto A, Bernardini D y Leon A (2004) Stearoylethanolamide exerts anorexic effects in mice via down-regulation of liver stearoyl-coenzyme A desaturase-1 mRNA expression. *FASEB J* **18**:1580–1582.

Thabuis C, Destaillats F, Landrier J-F, Tissot-Favre D y Martin J-C (2010) Analysis of gene expression pattern reveals potential targets of dietary oleoylethanolamide in reducing body fat gain in C3H mice. *J Nutr Biochem* **21**:922–928.

Thabuis C, Destaillats F, Lambert DM, Muccioli GG, Maillot M, Harach T, Tissot-Favre D y Martin J-C (2011) Lipid transport function is the main target of oral oleoylethanolamide to reduce adiposity in high-fat-fed mice. *J Lipid Res* **52**:1373–1382.

Timper K y Brüning JC (2017) Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity. *Dis Model Mech* **10**:679–689.

Tinoco AB, Nisembaum LG, Isorna E, Delgado MJ y de Pedro N (2012) Leptins and leptin receptor expression in the goldfish (*Carassius auratus*). Regulation by food intake and fasting/overfeeding conditions. *Peptides* **34**:329–335.

Tinoco AB, Armirotti A, Isorna E, Delgado MJ, Piomelli D y de Pedro N (2014a) Role of oleoylethanolamide as a feeding regulator in goldfish. *J Exp Biol* **217**:2761–2769.

Tinoco AB, Nisembaum LG, de Pedro N, Delgado MJ e Isorna E (2014b) Leptin expression is rhythmic in brain and liver of goldfish (*Carassius auratus*). Role of feeding time. *Gen Comp Endocrinol* **204**:239–247.

Tinoco AB, Valenciano AI, Gómez-Boronat M, Blanco AM, Nisembaum LG, de Pedro N y Delgado MJ (2015) Two cholecystokinin receptor subtypes are identified in goldfish, being the CCKAR involved in the regulation of intestinal motility. *Comp Biochem Physiol A Mol Integr Physiol* **187**:193–201.

Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Rev Fish Sci* **11**:107–184.

Tsang AH, Barclay JL y Oster H (2014) Interactions between endocrine and circadian systems. *J Mol Endocrinol* **52**:R1–R16.

Tsuboi K, Ikematsu N, Uyama T, Deutsch DG y Ueda AT y N (2013) Biosynthetic pathways of bioactive N-acylethanolamines in brain. *CNS Neurol Disord - Drug Targets* **12**:7–16.

Tsuboi K, Uyama T, Okamoto Y y Ueda N (2018) Endocannabinoids and related N-acylethanolamines: biological activities and metabolism. *Inflamm Regen* **38**:28.

Tutunchi H, Ostadrahimi A, Saghafi-Asl M y Maleki V (2019) The effects of oleoylethanolamide, an endogenous PPAR-α agonist, on risk factors for NAFLD: A systematic review. *Obes Rev* **20**:1057–1069.

U

Ueda N, Tsuboi K y Uyama T (2013) Metabolism of endocannabinoids and related N-acylethanolamines: Canonical y alternative pathways. *FEBS J* **280**:1874–1894.

V

Valenti M, Viganò D, Casico MG, Rubino T, Steardo L, Parolaro D y Di Marzo V (2004) Differential diurnal variations of anandamide and 2-arachidonoyl-glycerol levels in rat brain. *Cell Mol Life Sci CMLS* **61**:945–950.

Valenti M, Cottone E, Martinez R, de Pedro N, Rubio M, Viveros MP, Franzoni MF, Delgado MJ y Di Marzo V (2005) The endocannabinoid system in the brain of *Carassius auratus* and its possible role in the control of food intake. *J Neurochem* **95**:662–672.

van de Pol I, Flik G y Gorissen M (2017) Comparative physiology of energy metabolism: Fishing for endocrine signals in the early vertebrate pool. *Front Endocrinol* **8**:36.

Vanselow K y Kramer A (2007) Role of phosphorylation in the mammalian circadian clock. *Cold Spring Harb Symp Quant Biol* **72**:167–176.

Vatine G, Vallone D, Appelbaum L, Mracek P, Ben-Moshe Z, Lahiri K, Gothilf Y y Foulkes NS (2009) Light directs zebrafish *period2* expression via conserved D and E boxes. *PLoS Biol* **7**:e1000223.

Vatine G, Vallone D, Gothilf Y y Foulkes NS (2011) It's time to swim! Zebrafish and the circadian clock. *FEBS Lett* **585**:1485–1494.

Velarde E, Haque R, Iuvone PM, Azpeleta C, Alonso-Gómez AL y Delgado MJ (2009) Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic expression of *Period* and *Cryptochrome* transcripts in retina, liver, and gut. *J Biol Rhythms* **24**:104–113.

Velasco C, Librán-Pérez M, Otero-Rodiño C, López-Patiño MA, Míguez JM, Cerdá-Reverter JM y Soengas JL (2016a) Ghrelin modulates hypothalamic fatty acid-sensing and control of food intake in rainbow trout. *J Endocrinol* **228**:25–37.

Velasco C, Librán-Pérez M, Otero-Rodiño C, López-Patiño MA, Míguez JM y Soengas JL (2016b) Intracerebroventricular ghrelin treatment affects lipid metabolism in liver of rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* **228**:33–39.

Vera LM, de Pedro N, Gómez-Milán E, Delgado MJ, Sánchez-Muros MJ, Madrid JA y Sánchez-Vázquez FJ (2007) Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. *Physiol Behav* **90**:518–524.

Vera LM, Negrini P, Zagatti C, Frigato E, Sánchez-Vázquez FJ y Bertolucci C (2013) Light and feeding entrainment of the molecular circadian clock in a marine teleost (*Sparus aurata*). *Chronobiol Int* **30**:649–661.

Vieira E, Marroquí L, Batista TM, Caballero-Garrido E, Carneiro EM, Boschero AC, Nadal A y Quesada I (2012) The clock gene *Rev-erbα* regulates pancreatic β-cell function: Modulation by leptin and high-fat diet. *Endocrinology* **153**:592–601.

Vieira E, Marroquí L, Figueroa ALC, Merino B, Fernandez-Ruiz R, Nadal A, Burris TP, Gomis R y Quesada I (2013) Involvement of the clock gene *Rev-erb alpha* in the regulation of glucagon secretion in pancreatic alpha-cells. *PLoS One* **8**:e69939.

Vieira E, Merino B y Quesada I (2015) Role of the clock gene *Rev-erbα* in metabolism and in the endocrine pancreas. *Diabetes Obes Metab* **17**:106–114.

Vivas Y, Azpeleta C, Feliciano A, Velarde E, Isorna E, Delgado MJ y de Pedro N (2011) Time-dependent effects of leptin on food intake and locomotor activity in goldfish. *Peptides* **32**:989–995.

Volkoff H (2016) The neuroendocrine regulation of food intake in fish: A review of current knowledge. *Front Neurosci* **10**:540.

Volkoff H y Peter RE (2000) Effects of CART peptides on food consumption, feeding and associated behaviors in the goldfish, *Carassius auratus*: actions on neuropeptide Y- and orexin A-induced feeding. *Brain Res* **887**:125–133.

Volkoff H, Bjorklund JM y Peter RE (1999) Stimulation of feeding behavior and food consumption in the goldfish, *Carassius auratus*, by orexin-A and orexin-B. *Brain Res* **846**:204–209.

Volkoff H, Joy Eykelbosh A y Ector Peter R (2003) Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. *Brain Res* **972**:90–109.

Volkoff H, Unniappan S y Kelly SP (2009) The endocrine regulation of food intake. En: *Fish Neuroendocrinology* (Bernier NJ, Van der Kraak G, Farrel AP y Brauner CJ, eds), Academic Press Ltd, Londres, Reino Unido. pp 421–465.

W

Wall A y Volkoff H (2013) Effects of fasting and feeding on the brain mRNA expressions of orexin, tyrosine hydroxylase (TH), PYY and CCK in the Mexican blind cavefish (*Astyanax fasciatus mexicanus*). *Gen Comp Endocrinol* **183**:44–52.

Wang H (2008a) Comparative analysis of period genes in teleost fish genomes. *J Mol Evol* **67**:29–40.

Wang H (2008b) Comparative analysis of teleost fish genomes reveals preservation of different ancient clock duplicates in different fishes. *Mar Genomics* **1**:69–78.

Wang H (2009) Comparative genomic analysis of teleost fish *bmal* genes. *Genetica* **136**:149–161.

Wang M, Zhong Z, Zhong Y, Zhang W y Wang H (2015) The zebrafish Period2 protein positively regulates the circadian clock through mediation of retinoic acid receptor (RAR)-related orphan receptor α (Rorα). *J Biol Chem* **290**:4367–4382.

Wang N, Yang G, Jia Z, Zhang H, Aoyagi T, Soodvilai S, Symons JD, Schnermann JB, Gonzalez FJ, Litwin SE y Yang T (2008) Vascular PPARγ controls circadian variation of blood pressure and heart rate via activation of Bmal1. *Cell Metab* **8**:482–491.

Wang Q, Tikhonenko M, Bozack SN, Lydic TA, Yan L, Panchy NL, Mcsorley KM, Faber MS, Yan Y, Boulton ME, Grant MB y Busik JV (2014) Changes in the daily rhythm of lipid metabolism in the diabetic retina. *PLoS One* **9**:e95028.

Wei BQ, Mikkelsen TS, McKinney MK, Lander ES y Cravatt BF (2006) A second fatty acid amide hydrolase with variable distribution among placental mammals. *J Biol Chem* **281**:36569–36578.

Weikum ER, Liu X y Ortlund EA (2018) The nuclear receptor superfamily: A structural perspective. *Protein Sci* **27**:1876–1892.

Welch RD, Billon C, Valfort A-C, Burris TP y Flaveny CA (2017a) Pharmacological inhibition of REV-ERB stimulates differentiation, inhibits turnover and reduces fibrosis in dystrophic muscle. *Sci Rep* **7**.

Welch RD, Guo C, Sengupta M, Carpenter KJ, Stephens NA, Arnett SA, Meyers MJ, Sparks LM, Smith SR, Zhang J, Burris TP y Flaveny CA (2017b) Rev-Erb co-regulates muscle regeneration via tethered interaction with the NF-Y cistrome. *Mol Metab* **6**:703–714.

Wellner N, Tsuboi K, Madsen AN, Holst B, Diep TA, Nakao M, Tokumura A, Burns MP, Deutsch DG, Ueda N y Hansen HS (2011) Studies on the anorectic effect of N-acylphosphatidylethanolamine and phosphatidylethanolamine in mice. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids* **1811**:508–512.

Welsh DK, Takahashi JS y Kay SA (2010) Suprachiasmatic nucleus: cell autonomy and network properties. *Annu Rev Physiol* **72**:551–577.

White SL, Volkoff H y Devlin RH (2016) Regulation of feeding behavior and food intake by appetite-regulating peptides in wild-type and growth hormone-transgenic coho salmon. *Horm Behav* **84**:18–28.

Woldt E, Sebti Y, Solt LA, Duhem C, Lancel S, Eeckhoute J, Hesselink MKC, Paquet C, Delhaye S, Shin Y, Kamenecka TM, Schaart G, Lefebvre P, Nevière R, Burris TP, Schrauwen P, Staels B y Duez H (2013) Rev-erb-α modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy. *Nat Med* **19**:1039–1046.

Woods SC (2004) Gastrointestinal Satiety Signals I. An overview of gastrointestinal signals that influence food intake. *Am J Physiol-Gastrointest Liver Physiol* **286**:G7–G13.

Wu P, Li Y-L, Cheng J, Chen L, Zhu X, Feng Z-G, Zhang J-S y Chu W-Y (2016) Daily rhythmicity of clock gene transcript levels in fast and slow muscle fibers from Chinese perch (*Siniperca chuatsi*). *BMC Genomics* **17**.

Wu P, Bao L, Zhang R, Li Y, Liu L, Wu Y, Zhang J, He Z y Chu W (2018) Impact of short-term fasting on the rhythmic expression of the core circadian clock and clock-controlled genes in skeletal muscle of Crucian carp (*Carassius auratus*). *Genes* **9**:526.

X

Xu M y Volkoff H (2007) Molecular characterization of prepro-orexin in Atlantic cod (*Gadus morhua*): Cloning, localization, developmental profile and role in food intake regulation. *Mol Cell Endocrinol* **271**:28–37.

Xu M y Volkoff H (2009) Molecular characterization of ghrelin y gastrin-releasing peptide in Atlantic cod (*Gadus morhua*): Cloning, localization, developmental profile and role in food intake regulation. *Gen Comp Endocrinol* **160**:250–258.

Y

Yamamoto K, Ruuskanen JO, Wullimann MF y Vernier P (2011) Differential expression of dopaminergic cell markers in the adult zebrafish forebrain. *J Comp Neurol* **519**:576–598.

Yan A, Zhang L, Tang Z, Zhang Y, Qin C, Li B, Li W y Lin H (2011) Orange-spotted grouper (*Epinephelus coioides*) orexin: Molecular cloning, tissue expression, ontogeny, daily rhythm and regulation of NPY gene expression. *Peptides* **32**:1363–1370.

Yang X, Downes M, Yu RT, Bookout AL, He W, Straume M, Mangelsdorf DJ y Evans RM (2006) Nuclear receptor expression links the circadian clock to metabolism. *Cell* **126**:801–810.

Yang X, Lamia KA y Evans RM (2007) Nuclear receptors, metabolism, and the circadian clock. *Cold Spring Harb Symp Quant Biol* **72**:387–394.

Yang Z, Kim H, Ali A, Zheng Z y Zhang K (2017) Interaction between stress responses and circadian metabolism in metabolic disease. *Liver Res* **1**:156–162.

Yin L, Wu N, Curtin JC, Qatanani M, Szwergold NR, Reid RA, Waitt GM, Parks DJ, Pearce KH, Wisely GB y Lazar MA (2007) Rev-erbα, a heme sensor that coordinates metabolic and circadian pathways. *Science* **318**:1786–1789.

Yokobori E, Kojima K, Azuma M, Kang KS, Maejima S, Uchiyama M y Matsuda K (2011) Stimulatory effect of intracerebroventricular administration of orexin A on food intake in the zebrafish, *Danio rerio*. *Peptides* **32**:1357–1362.

Z

Zambrana-Infantes E, Rosell del Valle C, Ladrón de Guevara-Miranda D, Galeano P, Castilla-Ortega E, Rodríguez De Fonseca F, Blanco E y Santín LJ (2018) Palmitoylethanolamide attenuates cocaine-induced behavioral sensitization and conditioned place preference in mice. *Pharmacol Biochem Behav* **166**:1–12.

Zhang C, Forlano PM y Cone RD (2012) AgRP and POMC neurons are hypophysiotropic and coordinately regulate multiple endocrine axes in a larval teleost. *Cell Metab* **15**:256–264.

Zhang D, Tong X, Arthurs B, Guha A, Rui L, Kamath A, Inoki K y Yin L (2014) Liver clock protein BMAL1 promotes *de novo* lipogenesis through insulin-mTORC2-AKT signaling. *J Biol Chem* **289**:25925–25935.

Zhang J, Liu M, Zhang C, Li E, Fan M y Huang M (2018) Transcriptomic analyses of tributyltin-induced sexual dimorphisms in rare minnow (*Gobiocypris rarus*) brains. *Ecotoxicol Environ Saf* **156**:18–24.

Zhang R, Lahens NF, Ballance HI, Hughes ME y Hogenesch JB (2014) A circadian gene expression atlas in mammals: Implications for biology and medicine. *Proc Natl Acad Sci* **111**:16219–16224.

Zhang Z, Burch PE, Cooney AJ, Lanz RB, Pereira FA, Wu J, Gibbs RA, Weinstock G y Wheeler DA (2004) Genomic analysis of the nuclear receptor family: New insights into structure, regulation, and evolution from the rat genome. *Genome Res* **14**:580–590.

Zhao C y Gammie SC (2018) The circadian gene Nr1d1 in the mouse nucleus accumbens modulates sociability and anxiety-related behaviour. *Eur J Neurosci* **48**:1924–1943.

Zheng J-L, Luo Z, Zhu Q-L, Tan X-Y, Chen Q-L, Sun L-D y Hu W (2013) Molecular cloning and expression pattern of 11 genes involved in lipid metabolism in yellow catfish *Pelteobagrus fulvidraco*. *Gene* **531**:53–63.

Zhou C, Lei L, Deng X, Yuan D, Zhu C, Ye H, Luo H, Zhang C, Zhou J, Yang M, Wang J, Zeng B, Li B y Zheng Z (2019) Three forms of cocaine- and amphetamine-regulated transcript may be involved in food intake regulation in gibel carp (*Carassius auratus gibelio*). *Fish Physiol Biochem* **45**:921-933.

Zhu Q, Glazier BJ, Hinkel BC, Cao J, Liu L, Liang C y Shi H (2019) Neuroendocrine regulation of energy metabolism involving different types of adipose tissues. *Int J Mol Sci* **20**:2707.

Zimov S y Yazulla S (2004) Localization of vanilloid receptor 1 (TRPV1/VR1)-like immunoreactivity in goldfish and zebrafish retinas: Restriction to photoreceptor synaptic ribbons. *J Neurocytol* **33**:441–452.

Zvonic S, Ptitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, Wu X, Goh BC, Mynatt RL y Gimble JM (2006) Characterization of peripheral circadian clocks in adipose tissues. *Diabetes* **55**:962–970.